The fitness consequences of cellular immunity: studies with *Daphnia magna* and its sterilizing bacterial parasite

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DECLARATION

I declare that this thesis has been composed by myself and is entirely my own work, except for the collaborative input outlined below. No part of this thesis has been submitted to any other university in application for a higher degree.

Signed by Stuart K J R Auld

Chapter 2: Jennifer Scholefield, a Zoology Honours student, helped to collect data for this chapter and the resulting paper (published in *Proceedings of the Royal Society Series B*) was the result of a collaborative effort between Jennifer Scholefield, Tom Little and myself.

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THESIS ABSTRACT

Immune responses are presumed to contribute to host fitness, either by fighting off infections or *via* immunopathology. Research in this thesis sought to relate the magnitude of a putative immune response to infection and host and parasite fitness. The experiments and field studies presented here all focus on the interactions between the freshwater crustacean, *Daphnia magna* and its sterilizing bacterial endoparasite, *Pasteuria ramosa*, using the number of circulating haemocytes as a measure of host immune activity. I found substantial genetic variation in *Daphnia's* cellular response to *P. ramosa*, and that *Daphnia* genotypes that mount the strongest cellular responses are the most likely to get infected and suffer sterilization. Thus, a strong cellular response is associated with low, as opposed to high host fitness potential. There were also some host genotypes that mounted a weaker cellular response and did not go on to suffer infection, and some that lacked a cellular response and also never suffered infection with *P. ramosa.* These findings led to a heuristic two-stage model for infection, where the parasite has to (1) pass from the host gut to haemolymph and then (2) successfully overcome haemolymph-based immune effectors to reproduce and achieve fitness. I also demonstrate that both the magnitude of host cellular response and likelihood of infection increases with initial parasite dose in susceptible host genotypes, and that host cellular response is associated with likely infection under both host and parasite genetic variation. Parasitised *Daphnia* also have substantially more circulating haemocytes than their healthy counterparts in both the laboratory and in the wild, where there is substantial genetic and environmental variation. This is one of the very few examples of how an immune response designates low host and high parasite fitness potential in a

wild system. Finally, using a mixture of field study and common garden experiment, I demonstrate evolution in parasite infection traits over the course of an epidemic in a wild population, and that this evolution is associated with a decline in host abundance.

CHAPTER 1

General introduction, including an introduction to the model system

IMMUNE FUNCTION, INFECTION OUTCOME AND HOST-PARASITE COEVOLUTION

Immune systems: the arena of host-parasite interactions

By their very nature, parasites reduce the fitness of the hosts they infect. Host are not, however, willing victims, and have evolved defences to prevent infection or reduce the negative fitness impact of parasitism. Such defences include epithelia, phagocytosing cells, immune cytotoxins, lysozymes or antimicrobial peptides in animals (FRANK 2002; SCHMID-HEMPEL 2005), and waxy cuticles and *R* proteins in plants (JONES and DANGL 2006). Host immune defences are constantly being refashioned depending on the parasites they encounter (WATERHOUSE *et al.* 2007), as evidenced by rapid evolution in immune-related genes (OBBARD *et al.* 2006; SHIINA *et al.* 2006), and parasites also exhibit signs of rapid evolution in response to their changing hosts (CHEN and HOLMES 2006). The principal arena of host-parasite interaction is therefore often assumed to be the host's immune system, because to successfully infect a parasite must avoid, withstand or manipulate host immunological effectors. Only after overcoming these defences can the parasite reproduce and thus achieve fitness. So, in order to better understand how hosts and their parasites select against each other (and the subsequent coevolution), we must better appreciate the role of host immune functions. This is of prime interest for the many evolutionary ecologists of immune function (SCHMID-HEMPEL 2005).

Constitutive and inducible immune defences

Host defences are often divided into two broad categories: those that are constitutive, and those that are inducible. Constitutive defences operate all of the time, whereas inducible defences are only activated in the presence of a threat (*e.g.* an infectious parasite). Both categories of defence are costly in that they require resource investment for growth and maintenance, and these costs arise whether parasites are present or absent; however, inducible defences also incur activation costs (SHUDO and IWASA 2001). Haemolymph or blood-based immunological mechanisms are often inducible defences, for example, the insect antimicrobial peptide response or the mammalian cellular response (HOFFMANN 2003; JANEWAY *et al.* 1999). Their activation can therefore result in fitness costs to the host, *e.g.,* activation of the phenoloxidase cascade in *Tenebrio molitor* leads to damage to the host's malpighian tubules, an essential tissue for the regulation of host blood chemistry (SADD and SIVA-JOTHY 2006), and in humans, the multiple organ failure and low blood volume (septic shock) that can result from septicaemia are not directly caused by infecting bacteria, rather by the host's immune responses to those bacteria (MUNFORD 2006).

Host barrier mechanisms are often examples of constitutive defences against infectious agents *e.g.* the thick chitinous cuticle that encase invertebrates, or the waxy surface of plant leaves (BARNES and SIVA-JOTHY 2000; MARTIN 1964). Mealworm beetles (*Tenebrio molitor*) exhibit significant heritable genetic variation for cuticle colour, and beetles with darker cuticles are more resistant to entomopathogenic fungi (ARMITAGE and SIVA-JOTHY 2005). Some barrier defences do, however exhibit inducible characteristics: by thickening the peritrophic matrix (a component of the gut wall) *Aedes aegypti* mosquitoes can limit their burden of malarial parasites

(*Plasmodium gallinaceum* oocysts: BILLINGSLEY and RUDIN 1992). The nature of a host's immune defences will not only influence the fitness costs and benefits of immunity for the host, it will influence parasite fitness too: a barrier defence that prevents parasitic infection may have a very different impact on parasite fitness than a successful defence that operates within the host's haemocoel.

The fitness consequences of failed infections

The vast majority of epidemiological models stem from the host-microparasite and host-macroparasite models of Anderson and May, pioneers of the susceptible-infected-susceptible (SIS) and susceptible-infected-resistant (SIR) frameworks (ANDERSON and MAY 1978; ANDERSON and MAY 1979; ANDERSON and MAY 1981; MAY and ANDERSON 1978). However, when considering defence mechanisms, there are two reasons for non-infection: either (1) the parasite is neutralized by host immunological defences or; (2) the parasite failed to get past the host's barrier defences in the first place. The fitness consequences of these two types of failed infection (for both host and parasite) are potentially very different.

Parasites that successfully get past the host's barrier defences but fail to overcome the host's immunological defences are killed; they will not reproduce and achieve fitness. Whereas, parasites that fail to overcome the host's barrier defences may have the opportunity to infect a different host at a later time (*e.g.* a parasite that fails to penetrate an invertebrate's cuticle); in this case, fitness is delayed but not terminated. Failed infections are often thought to be an absolute dead-end for parasite fitness, however, in some cases, it may be better to fail at the barrier and infect a more

suitable host later than to overcome the barrier and be killed by haemocoel-based immunological defences. Further, in host-parasite systems where barrier defences are key in preventing infection, parasite selection on haemocoel-based defences will be minimal.

Interaction and variation: the importance of host and parasite genetics

The probability of infection is often highly dependent on the specific pairing of host and parasite genotypes. The infection phenotype thus depends on more than just the additive contributions of the host and parasite genomes (LAMBRECHTS 2010). This is termed genetic specificity, and has been documented in a number of host-parasite systems (CARIUS *et al.* 2001; SCHMID-HEMPEL and REBER 2004), but see (WILFERT and SCHMID-HEMPEL 2008), and can be detected using a two-way statistical analysis where it manifests as a host genotype-by-parasite genotype ($G_H \times G_P$) interaction (SCHMID-HEMPEL and EBERT 2003). Genetic specificity means that a parasite can only infect (and thus select against) a subset of available host genotypes, and that hosts can only resist (and thus select against) a subset of parasite genotypes. It can therefore maintain genetic variation in both host and parasite populations (BYERS *et al.* 2005). Genetic specificity is also a phenomenological outcome of models of host-parasite coevolution that rest on negative frequency-dependent selection (where being a rare genotype is advantageous: (HAMILTON 1980; HAMILTON *et al.* 1990b; JAENIKE 1978).

Whilst genetic specificity is a well-examined phenomenon, its mechanistic foundations are much less well understood (LAMBRECHTS 2010): indeed, there are often discrepancies between infection phenomena (both whether or not infection occurs in a

host and the extent of parasite burden) and variation in host immune activity. For example, invertebrate-parasite systems often exhibit strong genetic specificity for infection status (whether a host becomes infected or not) but invertebrate immune systems are believed to only be able to distinguish between broad classes of infectious agents (Gram positive bacteria, Gram negative bacteria and fungi: TZOU *et al.* 2002). Despite this gap between our understanding of molecular immunology and disease ecology, there is a paucity of studies that examine the relationship between host immune activity and genetic specificity. To date, Riddell *et al.*'s (2009) study is the only example. They found, in a bumblebee-trypanosome system, that the relative expression of four antimicrobial peptides depended on the specific combination of host and parasite genotypes (RIDDELL *et al.* 2009).

Aside from genetic specificity, the overall level of host and parasite genetic variation can strongly affect disease ecology. Elton (1958) noted that single varietal crop monocultures were more susceptible to devastating epidemics than multiple varietal cultivars, and more recent empirical studies using both plants and animals have shown disease to be less severe when host populations are genetically diverse (ALTERMATT and EBERT 2008; BAER and SCHMID-HEMPEL 1999; DWYER *et al.* 1997; ZHU *et al.* 2000). However, a genetically diverse host population is far more likely to contain at least some individuals that are susceptible to infection from a particular parasite. Indeed, recent theoretical work (that assumes the presence of genetic specificity) suggests that host genetic diversity does not reduce the likelihood of disease emergence, but can limit the severity of epidemics (LIVELY 2010). That study also shows that the rate of disease spread is inversely proportional to the number of host

genotypes and that highly infectious parasites would initially spread, but ultimately die out when the parasite exhausts susceptible hosts (LIVELY 2010).

Host-parasite coevolution: the possible role of host immune defences

Models of host-parasite coevolution fall into two broad classes: those that predict that antagonists engage in arms races of resistance and infectivity (*sensu* DAWKINS and KREBS 1979), and those that predict fluctuating selection between antagonists (*e.g.* the Red Queen Hypothesis: (HAMILTON 1980; JAENIKE 1978; VAN VALEN 1973), but see (FRANK 1994; GANDON *et al.* 2008; PARKER 1994; WOOLHOUSE *et al.* 2002). However, little is known of how host immune responses fit in with these models, specifically, whether or not they play a role in mediating host-parasite coevolution.

Arms race models suggest that the degree of adaptation will increase monotonically in both host and parasite populations: hosts are selected for better anti-parasite defences and parasites are selected for the ability to overcome host defences (WOOLHOUSE *et al.* 2002), leading to a series of selective sweeps (Figure 1.1A). There is evidence for this type of coevolution between the *Pseudomonas fluoresens* and its pathogenic phage Φ2, where there is directional selection on both host resistance and phage infectivity (BUCKLING and RAINEY 2002), as well as in numerous plants and their parasites (THOMPSON and BURDON 1992). Under this scenario, one would expect an increase in host resistance to be accompanied by an increase in the efficacy of a particular host immune function: the parasite would be selected by its ability to overcome this immune function until the costs of each trait (immunopathology or

energetic costs in the host and the cost of overcoming immunity in the parasite) outweigh the benefits.

Models incorporating fluctuating selection predict that reciprocal selection between hosts and their parasites will depend on the frequencies of individuals from interacting genotypes (HAMILTON 1980; HAMILTON *et al.* 1990a): hosts are selected to defend against the most common parasite, and parasites are selected to infect the most widely available host. This gives rise to negative frequency-dependent selection (Figure 1.1B). Fluctuating selection models rely on there being genetic specificity between host and parasite, which means that parasites can potentially (1) promote host diversity, and (2) track common host genotypes (DUFFY and FORDE 2009; JOKELA *et al.* 2009; WOOLHOUSE *et al.* 2002). There is evidence that parasites can track their hosts spatially: studies have demonstrated parasite-mediated selection against locally common genotypes natural field populations (DUNCAN and LITTLE 2007; DYBDAHL and LIVELY 1995; DYBDAHL and LIVELY 1998).

Fluctuating selection could result in two possible scenarios for immunological change: either (1) genetic specificity for infection outcome would be underpinned by genetic specificity for the efficacy of host haemocoel-based immunological mechanisms, as seen in the bumble bee-trypanosome system (RIDDELL *et al.* 2009). Alternatively, (2), genetic specificity for infection outcome may depend on the host barrier defences, in which case there may not be genetic specificity at the level of haemocoel-based defences, *i.e.* infection outcome will depend on a $G_H \times G_P$ interaction, but haemocoel-based immune efficacy may be described by a G_H and/or G_P main effect only. Thus, whether or not classical immune defences (based in the haemocoel) are

subject to arms race or fluctuating dynamics will depend on the involvement of barrier mechanisms in preventing parasitic infection.

Linking measures of host immune activity to both host and parasite fitness

It is frequently assumed that a strong host immune response will lead to increased immunity and thus increased host fitness in the face of parasitism. It is, however, becoming increasingly clear that this is often not the case: immune responses are often not limited to damaging infecting parasites; they can also cause damage to the host that mounts them (as discussed earlier). Immune activity can also be a marker for successful parasitic infection.

In cases where an immune response leads to successful host resistance, costs due to an overactive immune response may influence the relationship between parasite burden and host fitness (as discussed earlier). The association between host fitness and parasite burden may therefore be non-linear: hosts with a low parasite burden may be paying a high fitness cost for resistance and hosts with a high parasite burden may be paying a high fitness cost of parasitism (see Figure 1.2). Indeed, a non-linear relationship between parasite burden and fitness has been found in the blue tit *Cyanistes caeruleus* infected with the blood-parasite *Haemoproteus majoris*: birds with an intermediate parasite burden are most likely to survive the winter (STJERNMAN *et al.* 2008). Immunopathology is therefore a key player in the evolution of host immune mechanisms (*e.g.* the strength of immune response), as well as parasite virulence (reduction in host fitness due to parasitism: DAY *et al.* 2007; GRAHAM *et al.* 2005).

The efficacy of a particular host immune function may also depend on the number of infectious agents it faces, as increased parasite dose usually leads to an increased likelihood of infection (*e.g.* BEN-AMI *et al.* 2008b; BRUNNER *et al.* 2005; DE ROODE *et al.* 2007; OSNAS and LIVELY 2004). It is likely that an animal haemocyte will be able to phagocytose one invading bacterium, but is unlikely to be able to destroy a thousand bacteria. This is especially important because parasite transmission stages rarely exhibit a random distribution, especially when they produce a great many transmission stages at a time. Indeed, the majority of parasite transmission stages often originate from few hosts (ANDERSON and MAY 1991; EBERT *et al.* 2000). Therefore, the relationships between host immune activity, parasite resistance and host fitness (or indeed parasite fitness) are likely to be strongly influenced by the number of infective parasite transmission stages the host initially encounters.

The level of host immune activity, infection outcome and both host and parasite fitness are intrinsically interrelated traits. Measuring host immune function in isolation will, at best, give limited insight into ecological variation in immunity, and at worst, completely mislead (GRAHAM *et al.* 2011) and references therein). Immune mechanisms must therefore be linked to infection phenomena and fitness under realistic levels of parasite exposure, host genetic variation and parasite genetic variation before we can fully appreciate their ecological importance and any role they may play in mediating host-parasite coevolution (GRAHAM *et al.* 2011; LITTLE *et al.* 2005b; VINEY *et al.* 2005)

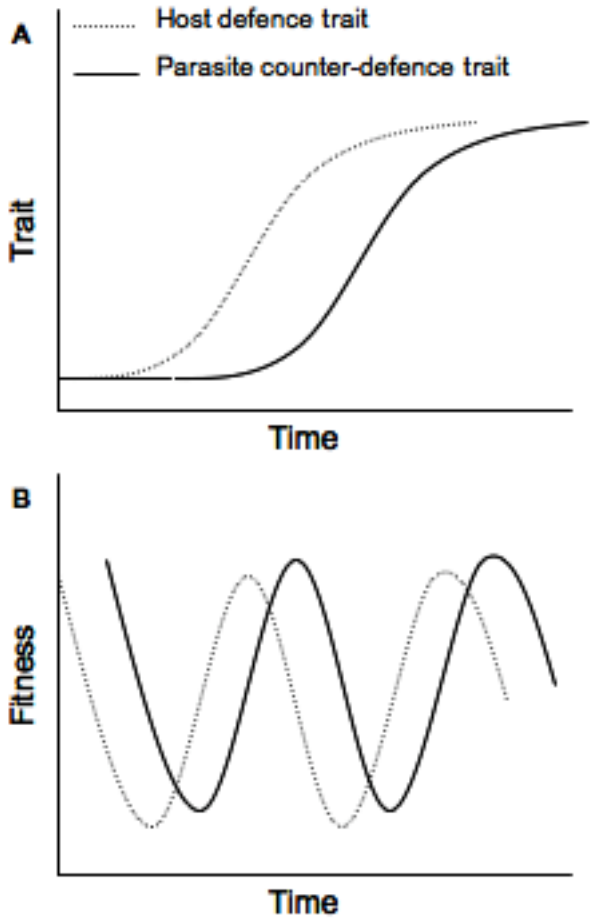

Figure 1.1. Coevolutionary dynamics arising from (A) arms race models and (B) fluctuating selection models.

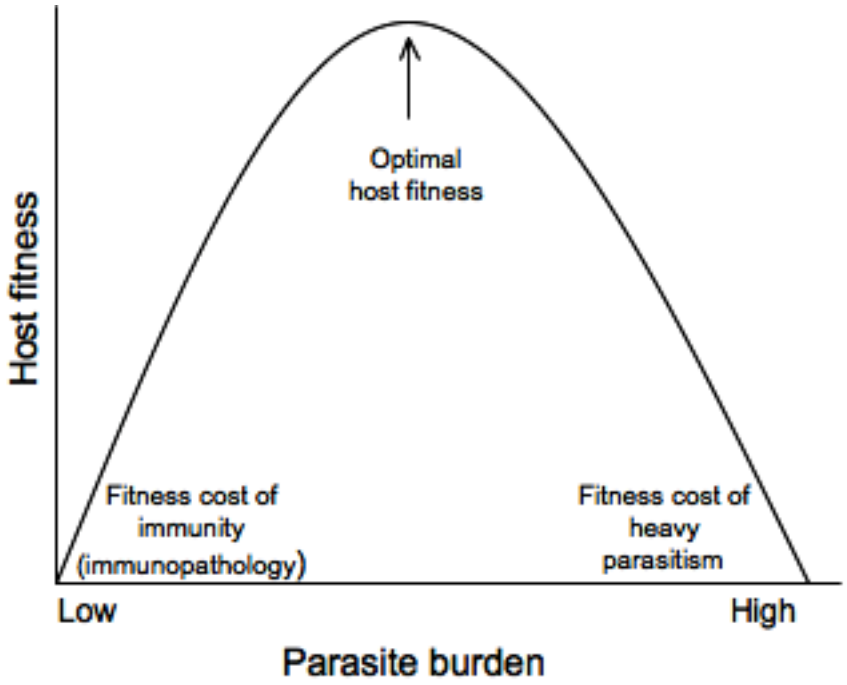

Figure 1.2. Potential relationship between host fitness and parasite burden. Minimizing parasite burden does not necessarily lead to maximum host fitness because of the costs of mounting an immune response.

THESIS AIMS

This thesis examines the relationships between host immune activity, infection status (infected or not) and both host and parasite fitness using a naturally coevolving host-parasite system: the crustacean *Daphnia magna* and its sterilizing bacterial parasite, *Pasteuria ramosa.* The relative contributions of environment (*e.g.* food levels, temperature *etc.*) and genetics (host and parasite) to infection outcome in the *Daphnia-Pasteuria* system have been well explored (see BEN-AMI *et al.* 2010; BEN-AMI *et al.* 2008b; LITTLE and EBERT 1999; LITTLE and EBERT 2000; MITCHELL *et al.* 2005; VALE and LITTLE 2009; VALE *et al.* 2008). However, far less is known about the role of the host's immune system in *Daphnia-Pasteuria* interactions (*c.f.* LABBÉ and LITTLE 2009; MUCKLOW *et al.* 2004).

Nevertheless, our limited knowledge of *Daphnia* immunology does have a long history: one of the fathers of modern immunology, Nobel Laureate Ilya Metchnikoff found that *Daphnia* have amoeboid haemocytes that are mobilised in response to a yeast-like fungal parasite (now known to be *Metschnikowia bicuspidata*) (METCHNIKOFF 1884). Haemocytes are known to be important immunological weapons for a number of invertebrate hosts, protecting against a number of single- and multi-cellular parasites. *Drosophila* haemocytes phagocytose bacteria such as *Escherichia coli* (ELROD-ERICKSON *et al.* 2000) and encapsulate parasitoid wasp larvae such as *Asobara tabida* (SALT 1970). The baseline number of circulating haemocytes is a good predictor of host *Drosophila* resistance to parasitoid infection (ESLIN and PRÉVOST 1998; KRAAIJEVELD *et al.* 2001). The mollusc *Biomphalaria glabrata* also encapsulates sporocysts of the blood-fluke *Echinostoma caproni* with its haemocytes (ATAEV and

COUSTAU 1999), and resistant genotypes of *B. glabrata* mount a larger cellular response than their susceptible counterparts. Haemocytes are play a key role in blood clotting in the horseshoe crab, *Limulus polyphemus* (ARMSTRONG and ARMSTRONG 2003; ISAKOVA and ARMSTRONG 2003), which is essential for both wound-healing and for the binding and killing of bacteria within the host's haemocoel (see THEOPOLD *et al.* 2004 for a review); they are also known to generate potent cytotoxic chemicals such as reactive species of oxygen and nitrogen in order to kill encapsulated parasites (see NAPPI and OTTAVIANI 2000).

Although Metchnikoff's early work suggests haemocyte mobilization as a possible mechanism for *Daphnia magna'*s defence against parasites, there have hitherto been no attempts to link this immune measure to fitness consequences in either the *Daphnia* or any of its infecting parasites. The *Daphnia-Pasteuria* system is therefore excellently poised to examine the possible role cellular immune responses in mediating host and parasite fitness under both genetic and environmental variation; this is the general aim of my thesis.

In Chapter 2, I determine: (1) the timing of the *Daphnia* cellular response to *P. ramosa*, (2) the genetic variation for the presence and magnitude of host cellular response and how this relates to *Daphnia* susceptibility to *P. ramosa,* and (3) whether it is the presence of *P. ramosa* spores or the process of infection that results in a cellular response in the host. The experiment in Chapter 3 examines how the association between *Daphnia* haemocyte number and both host and parasite fitness changes with increasing initial parasite dose; specifically, whether the efficacy of the *Daphnia* cellular response depends on the number of infectious *P. ramosa* spores it encounters.

As discussed earlier, the *Daphnia-Pasteuria* system is known for strong genetic specificity, both in terms of whether or not an infection occurs and the fitness consequences of infection for both host and parasite (CARIUS *et al.* 2001; VALE and LITTLE 2009). In Chapter 4, I examine whether genetic specificity for infection outcome (probability of infection, host offspring production and parasite transmission spore production) corresponds to genetic specificity for the number of *Daphnia* circulating haemocytes, *i.e.* whether the number of circulating haemocytes is predictive of infection outcome over both host and parasite genetic variation (*sensu* RIDDELL *et al.* 2009).

In Chapter 5, I examine whether associations between immune activity and disease phenomena in the laboratory are observable in the wild, where both biotic and abiotic environmental variation is abundant. Chapter 6 takes a similar approach, testing whether parasite fitness traits (in terms of the parasite's ability to infect the host and its reproductive success once infection has occurred) change over the course of an epidemic; it also examines the possible role of host cellular immunity in mediating these parasite fitness traits.

THE MODEL: *DAPHNIA MAGNA* AND ITS STERILIZING MICROPARASITE, *PASTEURIA RAMOSA*

The host: Daphnia magna

Daphnia magna Strauss is a small, planktonic crustacean that resides in still, freshwater bodies. Like many other Branchiopods it is a filter feeder, with single-celled algae

forming bulk of its diet. *Daphnia magna* is cyclically parthenogenetic: it reproduces asexually in the main (by apomictic parthenogenesis) but has sex when there are changes in certain environmental conditions, including photoperiod, food quality/quantity and temperature, or the presence of fish kairomones (CARVALHO and HUGHES 1983; HOBAEK and LARSSON 1990; KLEIVEN *et al.* 1992).

When conditions favour sex, females produce males mitotically, or haploid eggs meiotically. The males go on to produce sperm by meiosis, and the subsequent sexual union of these gametes results in a female resting egg. Resting eggs are encased in a freezing and desiccation-resistant casing, called an ephippium, which protects the offspring from harsh environmental conditions, sometimes for many years (DECAESTECKER *et al.* 2007). Usually there are two resting eggs per ephippium, and these eggs contain true sisters (with a relatedness of 0.5). When reproducing parthenogentically, female *Daphnia* produce 1-100 eggs after each moult. These eggs develop into offspring in the brood chamber over a period of four to eight days, after which they are released. A well-fed adult female can produce a clutch every three days.

The parasite: Pasteuria ramosa

Daphnia magna are infected by a multitude bacterial, fungal and microsporidian parasites (EBERT 2005; EBERT 2008; GREEN 1974). All of the studies in this thesis focus on a particularly well-described parasite of *D. magna*: *Pasteuria ramosa* Metchnikoff 1888. *Pasteuria ramosa* is a spore-forming obligate endoparasite of *D. magna* (as well as other Cladocerans), and is a close relation to the nematode parasite, *Pasteuria penetrans* (EBERT *et al.* 1996). Its transmission spores lie at the bottom of

ponds and are taken up when *Daphnia* filter their food. Once in the *Daphnia* haemolymph, the spores begin their life cycle, culminating in millions more transmission spores, which are released when the host dies (see Figure 1.3A). Transmission of parasite spores is purely horizontal, from the cadavers of dead, previously infected hosts. Like the resting eggs of the *Daphnia, Pasteuria* transmission spores are highly resistant to environmental stress; spores from sediment cores can remain infective, despite being decades old (DECAESTECKER *et al.* 2007; DECAESTECKER *et al.* 2004).

Pasteuria-infected *Daphnia* show disease symptoms 15-25 days after exposure to the parasite, and they are easily diagnosed by eye: infected *Daphnia* often grow larger, and their haemolymph acquires a dark-red colouration (see Figure 1.3B). Also, infected *Daphnia* usually become completely sterile (although in some very rare cases, individuals continue to reproduce at a very low level). Infected *Daphnia* live 20-60 days in laboratory conditions, while uninfected *Daphnia* can live for 90 days or even longer. Since *P. ramosa* is obligate to *Daphnia,* the spore solutions used in the experiments in this thesis were obtained from the macerated cadavers of previously infected hosts.

Why use Daphnia and Pasteuria?

Numerous researchers have used *Daphnia* to better understand population dynamics (CARVALHO 1987; CARVALHO and CRISP 1987; HEBERT 1978), predator-prey dynamics (BOERSMA *et al.* 1998; LASS and BITTNER 2002; SLARSARCZYK *et al.* 2005), reproductive strategies (DUNCAN *et al.* 2006; INNES *et al.* 2000; INNES and SINGLETON

2000) and host-parasite interactions (EBERT 2008). *Daphnia* reproductive biology makes them particularly amenable as experimental models – by keeping female *Daphnia* in good conditions (without any sexual cues), it is possible to maintain independent clonal lines. Such control over genetic variation allows one to conduct very powerful experiments. Indeed, many life-history experiments have used *Daphnia* because it is possible to effectively partition the contributions of environment and genotype to a given phenotype (*e.g.* EBERT 1991; EBERT 1993; LYNCH 1984).

This system has many other virtues. First, it is easy to control host exposure to parasites. In many host-parasite models, there is often a worry about cross-infection with airborne pathogens, but with the *Daphnia-Pasteuria* system one can have a jar containing a high dosage of spores next to a control jar and not worry at all about cross-contamination (EBERT 2005). Second, *Pasteuria ramosa* is a natural parasite of *Daphnia magna*, and the fact that it infects via the gut means that one does not need to use artificial techniques to introduce it to the host, *i.e.* injection. Third, the *Daphnia-Pasteuria* system often occurs as a single host-single parasite system in nature, meaning that one can twin controlled laboratory experiment with examination of disease phenomena in the field. Sediment samples containing *Pasteuria* spores and *Daphnia* ephippia can be brought into the laboratory, the spores can be frozen at -20°C and the ephippia can be hatched out. One can therefore easily perform controlled laboratory experiments on wild-collected organisms.

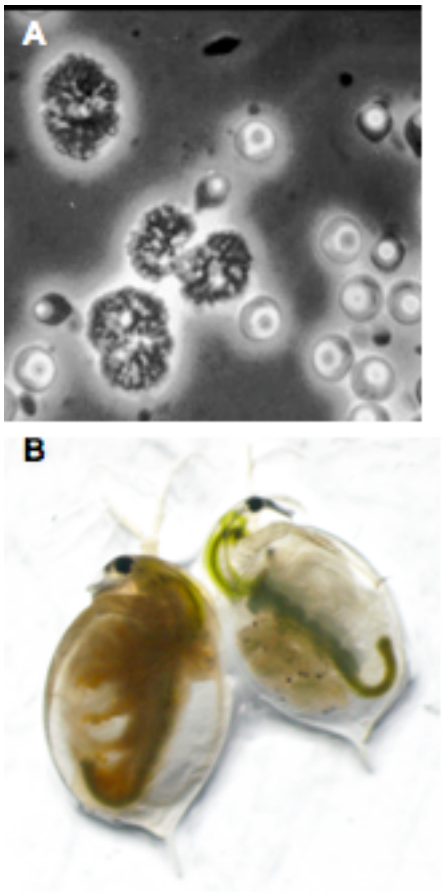

Figure 1.3. (A) the developmental stages of *Pasteuria ramosa,* including large cauliflower stages and smaller final-stage transmission spores. The transmission spores are approximately 3-6 μm in diameter. (B) two adult *Daphnia magna*. These *Daphnia* are the same genotype (clone) and the same age, but the one on the left is infected with *P. ramosa* while the one on the right is healthy (note the presence of offspring in the brood chamber).

CHAPTER 2

Genetic variation in the cellular response of *Daphnia magna* (Crustacea: Cladocera) to its bacterial parasite

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Linking measures of immune function with infection, and ultimately, host and parasite fitness, is a major goal in the field of ecological immunology. In this study, I tested for the presence and timing of a cellular immune response in the crustacean *Daphnia magna* following exposure to its sterilising endoparasite *Pasteuria ramosa*. I found that *D. magna* possesses two cell types circulating in the haemolymph: a spherical one, which I call a granulocyte, and an irregular-shaped amoeboid cell first described by Metchnikoff over 125 years ago. *Daphnia magna* mounts a strong cellular response (of the amoeboid cells) just a few hours after parasite exposure. I further tested for, and found, considerable genetic variation for the magnitude of this cellular response. These data fostered a heuristic model of resistance in this naturally coevolving host-parasite interaction. Specifically, the strongest cellular responses were found in the most susceptible hosts, indicating resistance is not always borne from a response that destroys invading parasites, but rather stems from mechanisms that prevent their initial entry. Thus, *D. magna* may have a two-stage defence – a genetically determined barrier to parasite establishment, and a cellular response once establishment has begun.

INTRODUCTION

Parasites often impose substantial costs on their hosts, as evidenced both by the severe effects they can have on individuals, and in the impact they may have on host population sizes (DUNCAN and LITTLE 2007; HUDSON *et al.* 1998; VAN ALFEN *et al.* 1975). Host defence mechanisms therefore make a key contribution to organismal fitness, and genetic variation for these mechanisms may contribute to host evolution in the face of parasitism. The first line of defence for the invertebrate host often consists of the barrier defences of the cuticle, or more complex defences of the gut epithelium (ARTIS 2008). After these come the haemolymph-based immune defences, for example phagocytic haemocytes, antimicrobial peptides, or lysozymes (HOFFMANN 2003; MYDLARZ *et al.* 2006). Much of our understanding of invertebrate immunity is built on studies of insect-parasite systems, although there are notable exceptions (MYDLARZ *et al.* 2006). I argue the importance of strengthening our knowledge of invertebrate immunity beyond the insects, as well as the need to develop deep understanding of the interplay between naturally coevolving antagonists.

One of the goals of ecological immunology is to determine the role immunological mechanisms play in mediating variation in fitness when organisms are exposed to parasites. To address the function that immune responses have in determining infection outcomes and, ultimately, the fitness consequences of infection (or self-harm due to immunopathology), it is necessary to measure how immune effector systems vary under genetic and environmental variation. However, many studies aiming to elucidate immune mechanisms have done so in the absence of pathogens, under controlled laboratory conditions and in homogeneous, inbred

genetic backgrounds. Thus, while providing the necessary mechanistic backbone for studying immune function, this approach does not address variation in natural populations (LITTLE *et al.* 2005b). However, a considerable body of evidence suggests that the impact of genetic and environmental variation on infection is substantial (LAZZARO and LITTLE 2009; MYDLARZ *et al.* 2006), and it is thus difficult to extrapolate from laboratory measures of immune responsiveness to variation in fitness (VINEY *et al.* 2005).

Here, I tested for a cellular immune response in a naturally coevolving host-parasite model: the aquatic crustacean, *Daphnia magna* and its sterilising bacterial endoparasite, *Pasteuria ramosa.* The fitness consequences, for example host sterilisation or mortality, of *P. ramosa* infection have been extensively studied under genetic and environmental variation (DUNCAN *et al.* 2006; MITCHELL *et al.* 2005; VALE and LITTLE 2009; VALE *et al.* 2008), but the mechanisms of resistance have received less attention in this system (LABBÉ *et al.* 2009; MUCKLOW and EBERT 2003; MUCKLOW *et al.* 2004). Circulating haemocytes are an important anti-parasite defence in many invertebrates (ATAEV and COUSTAU 1999; CANESI *et al.* 2002; COTTER *et al.* 2004; ELROD-ERICKSON *et al.* 2000; KRAAIJEVELD *et al.* 2001), and have been found in *D. magna* (METCHNIKOFF 1884). They are central to the innate immune system, being involved in phagocytosis and encapsulation; they are also vehicles for other immune functions, *e.g.* the generation of reactive oxygen and nitrogen species, as well as antimicrobial peptides and phenoloxidase (PO: (STRAND 2008). For these reasons, I chose them as the immune marker for this study. Both the induction of a cellular response and its magnitude are likely to contribute to host fitness when the host is in the presence of parasites.

This study also examines how magnitude of cellular response varies across multiple host genotypes. By embracing host genetic variation, I hope to gain further insight into how parasitism could influence host genetic structure, and ultimately, host evolution. I also test how infection outcome differs across host genotypes, allowing us to link our measures of cellular response with susceptibility. Finally, I sought to determine whether it is the mere presences of parasite spores in the gut, or the process of spores moving from gut to haemolymph that elicits a cellular response in the host.

MATERIALS AND METHODS

Host and parasite organisms

Daphnia magna is a freshwater crustacean of shallow, eutrophic ponds. It reproduces by cyclical parthenogenesis, where apomictic parthenogenesis is the main reproductive mechanism, but bouts of sexual reproduction occur in the presence of specific cues (CARVALHO and HUGHES 1983; HOBAEK and LARSSON 1990; SLARSARCZYK *et al.* 2005). By keeping *D. magna* in the absence of sexual cues, purely clonal lines can be maintained in the laboratory.

Pasteuria ramosa is a spore-forming, bacterial endoparasite, obligate to *D. magna*. It is transmitted horizontally from dead, infected hosts (EBERT *et al.* 1996), and is believed to infect *via* the gut and proliferate in the host's haemolymph. Successful *P. ramosa* infections have a profound impact on host fitness, often causing complete host sterilization and premature death (EBERT *et al.* 1996).

Twelve of the 16 host genotypes used here were founded from single animals, hatched from individual ephippia (sexually produced resting eggs) in the laboratory. Ephippia were from pond mud collected in Gaazerfeld, Germany in 1997. The other four genotypes (numbers 3, 4, 7 and 13) were also founded from single individuals, but these were collected as adults from Gaazerfeld in 1997 and have since been kept in a state of clonal reproduction. The *P. ramosa* isolate originated from an single infected *D. magna* from that same pond (CARIUS *et al.* 2001), and has been used in a variety of experiments since that time. The *P. ramosa* spore solution used here was made by homogenising previously infected hosts with ddH_2O.

Experimental setup

Independent replicates for each *D. magna* genotype were maintained for three generations to minimise variation in condition. Animals were kept in jars containing 200 ml of artificial medium (KLUTTGEN *et al.* 1994) modified using one twentieth of the recommended SeO_2 concentration (EBERT *et al.* 1998) and fed 5.0 ABS *Chlorella vulgaris* algal cells per day (ABS is the optical absorbance of 650 nm white light by the *Chlorella* culture). Their medium was refreshed three times per week. There were five *Daphnia* per jar and jars were incubated at 20°C on a 12L:12D light cycle. The second-clutch neonates from the third generation were used in each of the four experiments.

The first experiment examined host cellular response in four host clones, or genotypes. For this four-genotype cell experiment, replicates were allocated to one of two parasite treatments: non-exposed or parasite-exposed. Parasite treatment

lasted for 2 hrs, 4 hrs, 6 hrs or 8 hrs. Thus, there were six replicates per genotype, per parasite treatment, per time treatment. The second and third experiments both studied 16 genotypes: the second experiment examined host cellular response and the third experiment measured infection outcome. Like the previous four-genotype cell experiment, replicates were allocated to one of two parasite treatments (non-exposed or parasite-exposed), however all replicates were exposed for the same amount of time: five hours. There were six and twelve replicates per parasite treatment, per genotype for the second and third experiment, respectively. Finally, a fourth experiment used one genotype (genotype 4 from the previous experiment) to test for the presence of a cellular response when the host was exposed to killed (non-infective) spores or live (infective) spores. Spores were killed by heating them in a water bath at 95ºC for 30 minutes. Replicates were allocated to three treatments: non-exposed and parasite-exposed, and exposed to killed parasites. There were 8 replicates per treatment.

Parasite exposures were carried out as follows. When at least 3 out of 5 of the *Daphnia* in a replicate had deposited eggs in their brood chamber, the replicate was exposed to its parasite treatment. The 5 *Daphnia* of the replicate were placed together in a well of a 24-well cell plate (Costar, Corning Inc., NY). Parasite-exposed replicates received 50 000 *P. ramosa* spores from the pre-prepared solution. Non-exposed control replicates received the same concentration of uninfected *D. magna* homogenised in ddH_2O.

Haemocyte collection and counting

After parasite treatment, five *Daphnia* from each replicate were placed in a cell extraction chamber containing 4.0 μl of ice-cold anticoagulant buffer (98 mM NaOH, 186 mM NaCl, 17 mM EDTA and 41 mM citric acid, pH adjusted to 4.5: LAVINE *et al.* 2005). A 25-guage needle (BD Microlance, Drogheda, Ireland) was used to pierce the *Daphnia* heart, causing haemolymph to pool into the medium. The *Daphnia* were then removed, and the haemolymph solution was mixed thoroughly using a pipette. Four μl of the cell suspension were placed in a fertility counting chamber [0.001 mm^2 x 0.100 mm (depth)] (Hawksley, Lancing, Sussex, UK), and the number of amoeboid haemocytes was counted (Figure 2.1). The number of granulocytes did not vary between treatments in any of the cell experiments and are not discussed further. Haemocyte counts were converted to number of cells per microlitre of haemolymph-buffer solution.

Life history assays

After parasite treatment, one of the five *Daphnia* from each replicate of the 16-clone life history experiment was randomly selected and kept individually in 60 ml of artificial medium and fed 1.0 ABS *C. vulgaris* cells per day. Their medium was refreshed three times per week, or after the *Daphnia* had a clutch of offspring, and jars were incubated at 20˚C on a 12L:12D light cycle. Jars were checked daily for clutches and the number of offspring was recorded at each clutch. From day twenty-five post parasite exposure, hosts were examined for symptoms of *P. ramosa* infection. Symptoms include cessation of reproduction, absence of ovaries and bacterial growth in the haemolymph. The experiment ran for 32 days.

Statistical analyses

Data were analysed using R (IHAKA and GENTLEMAN 1996; R 2005). To achieve normality of distribution in the data, haemocyte counts were log-transformed for the four genotype and 16-genotype cell experiments and square-root transformed for the killed parasite cell experiment. For the four-genotype cell experiment, I tested fixed effects of host genotype, parasite treatment and exposure time, as well as all interaction terms. For the 16-genotype cell experiment, I tested the fixed effects of host genotype and parasite exposure, along with their interaction. Welch's two sample *t*-tests were performed *post hoc* on the 16-genotype cell data to test for the presence of a significant cellular response in each of the host genotypes, and the results were corrected for multiple comparisons (HOLM 1979). For the killed parasite experiment, I tested for differences between parasite-exposure treatments.

I report the full statistical models for both the four-genotype and 16-genotype cell data, along with the proportion of the variance explained by each of the terms in the full model. Variance proportions were calculated by dividing the sequential sum of squares for each term by the total sum of squares for the model. I then multiplied these proportions by 100 to find the percentage variance explained by each term.

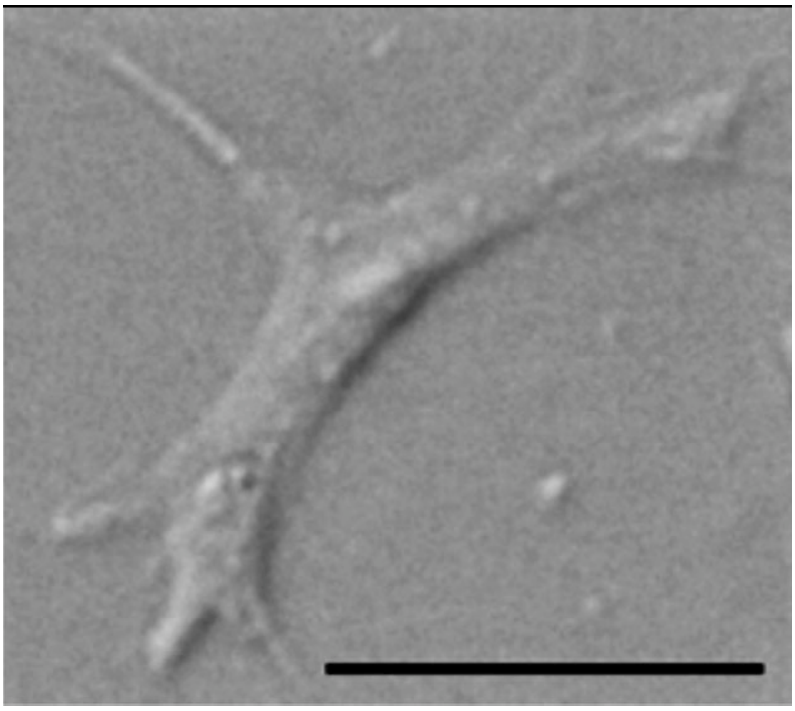

Figure 2.1. Differential interference contrast image of an amoeboid haemocyte from *D. magna.* Scale bar, 5 mm.

RESULTS

Four-genotype cell experiment

Haemocyte counts were obtained from 240 *Daphnia* from 48 jars. Averaging across all genotypes, mean circulating haemocyte number per microlitre from the *P. ramosa*-exposed replicates was 599 ± 80 (n = 24), whereas control replicates had a mean of 196 ± 11 circulating haemocytes (n = 24). However, the magnitude of the parasite-induced cellular response depended on the identity of the host genotype: *i.e.* there was a parasite exposure by host genotype interaction (see Figure 2.2, Table 2.1). When genotype is coded as a random effect, parasite exposure remains significant ($F_{1,3}$ = 15.26, $p < 0.05$), and a model with the parasite exposure-by-genotype effect explained significantly more variation than did a model without the interaction term ($\chi^2 = 4.60$, d.f. = 1, $p < 0.05$).

16-genotype cell experiment

Haemocyte counts were obtained from 960 *Daphnia* from 192 jars. As before, a cellular response followed *P. ramosa* exposure, with a mean per microlitre haemocyte count that was highly consistent with the previous experiment: 614 ± 50 cells for *P. ramosa*-exposed replicates (n = 96) and 208 ± 17 haemocytes per microlitre for control jars (n = 96). Basal haemocyte counts differed across host genotypes ($F_{15,80}$ = 4.49, $p < 0.001$: Figure 2.3); and, there was also considerable genetic variation in the magnitude of cellular response, varying between a one and nine-fold increase in haemocyte number depending on the identity of the host genotype (Figure 2.4). Statistically, this appears as a strong parasite exposure by

host genotype interaction (Table 2.2). The three host genotypes that mounted the strongest cellular response were the three genotypes that suffered infection from *P. ramosa* (Figure 2.4). Again, the parasite treatment remains significant with genotype as a random effect ($F_{1,15} = 27.76$, $p < 0.0001$), and the parasite exposure-by-genotype effect explained significantly more variation than did a model without the interaction term ($\chi^2 = 32.86$, d.f. = 1, $p < 0.0001$).

Post hoc tests revealed a significant cellular response, *i.e.* that the number of circulating haemocytes was greater in exposed *versus* unexposed in the following five host genotypes: 3, 4, 17, 20 and 22 (Figure 2.4). This was after the data were corrected using the sequential Bonferroni adjustment (HOLM 1979). Of these five responding genotypes, three suffered infection from *P. ramosa* (3, 4 and 17).

16-genotype life history experiment

Successful infection was recorded in three of the 16 genotypes, where infection with *P. ramosa* caused a substantial reduction in the number of offspring produced by the *Daphnia*. Of replicates from the parasite-exposed treatment, uninfected hosts had 48.05 ± 0.78 offspring, whereas infected hosts had 32.21 ± 1.15 offspring ($t = 11.35$, d.f. = 47.61, $p < 0.0001$).

Killed parasite cell experiment

Haemocyte counts were obtained from 120 *Daphnia* from 24 jars. The strongest cellular response followed exposure to live parasite spores, with a mean haemocyte count of 584 ± 83 haemocytes per microlitre for live *P. ramosa*-exposed jars ($n = 8$)

and 65 ± 13 for control jars (n = 8). There was also a smaller but significant cellular response from jars exposed to heat-treated *P. ramosa* spores: 238 ± 30 haemocytes (n = 8). *Post hoc* tests revealed that haemocyte counts from all treatments were significantly different from each other (Tukey's HSD, $p < 0.05$). Only jars exposed to live *P. ramosa* spores went on to develop infection.

Table 2.1. Summary of analysis of the number of circulating haemocytes in an experiment involving four host genotypes of *D. magna.* The effects tested were parasite (exposed or not), time post-exposure and host genotype.

number of haemocytes	DF	*F*	*p*	% var [a]
time	3	2.18	0.09	2.19
parasite	1	61.31	**< 0.0001**	20.57
genotype	3	11.13	**< 0.0001**	11.2
time x parasite	3	1.82	0.14	1.84
time x genotype	9	1.09	0.37	3.29
parasite x genotype	3	4.02	**< 0.01**	4.05
time x parasite x genotype	9	1.05	0.40	3.18
error	160			53.69

[a] Percentage of the total variance explained by each term in the full model.

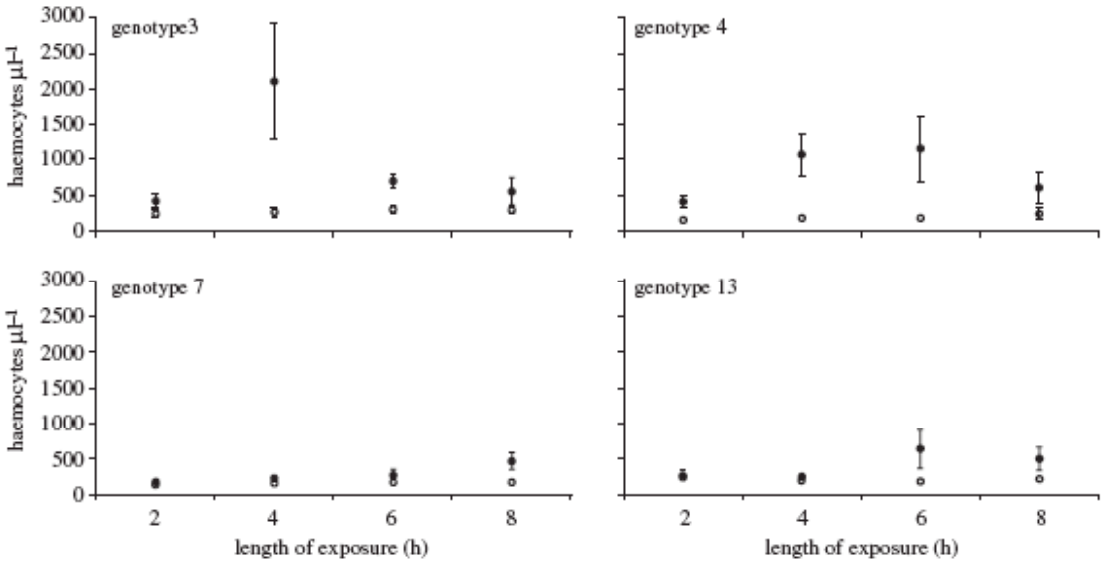

Figure 2.2. Haemocyte counts per host in *P. ramosa*-exposed and control *D. magna* (filled and open symbols, respectively; $n = 6$ and each replicate consists of 5 *Daphnia*). Error bars are 1 s.e.m. See Table 2.1 for statistical details.

Table 2.2. Summary of analysis of the number of circulating haemocytes in an experiment involving 16 host genotypes of *D. magna.* The effects tested were parasite (exposed or not) and host genotype.

number of haemocytes	DF	*F*	*p*	% var [a]
parasite	1	157.29	**< 0.0001**	28.53
genotype	15	9.72	**< 0.0001**	26.67
parasite x genotype	15	5.67	**< 0.0001**	15.54
error	160			29.26

[a] Percentage of the total variance explained by each term in the full model.

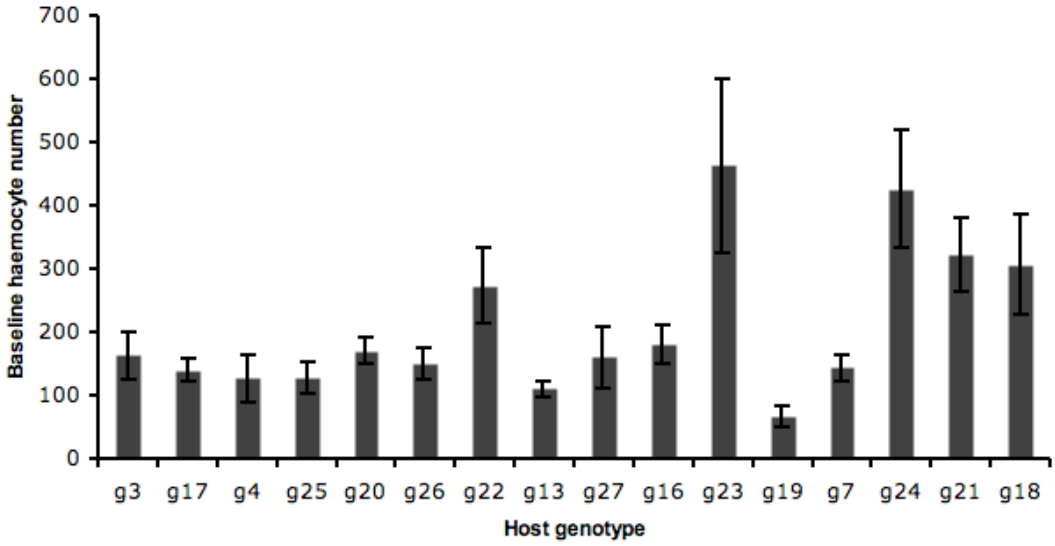

Figure 2.3. Baseline haemocyte numbers non parasite-exposed *D. magna* (n = 6, each replicate consists of 5 *Daphnia*).

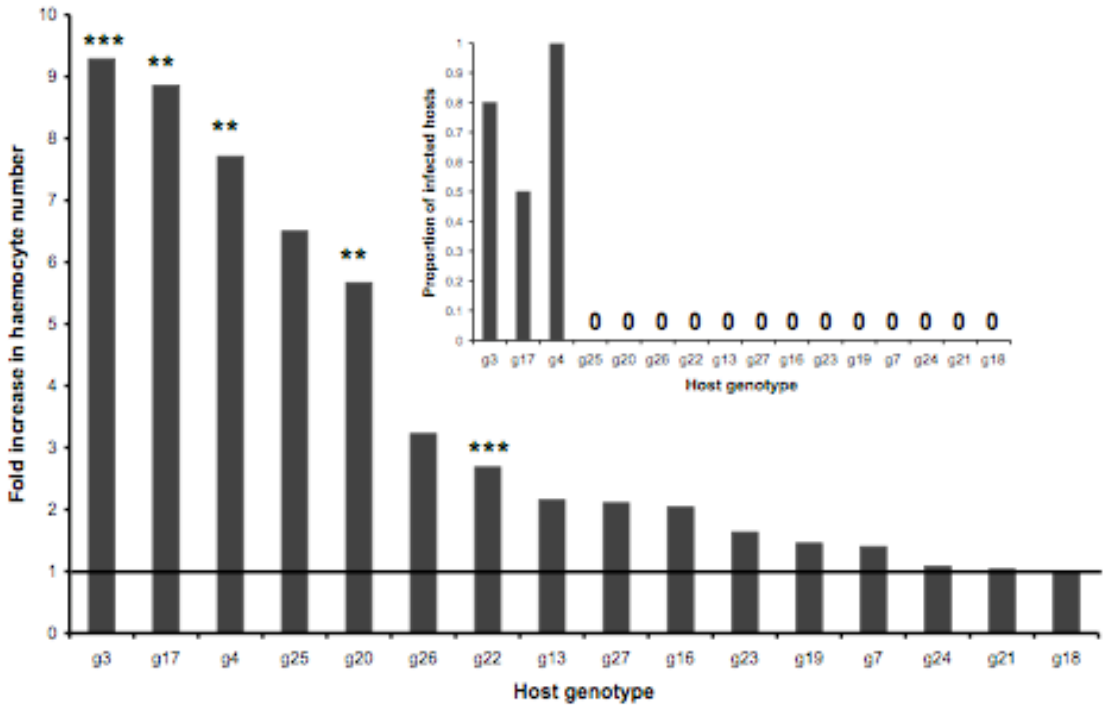

Figure 2.4. Fold induction of haemocyte numbers in *P. ramosa*-exposed *D. magna* (n = 6, each replicate consists of 5 *Daphnia*), relative to unexposed *D. magna* (n = 6, each replicate consists of 5 *Daphnia*). The bold line at y = 1 shows the uninduced (basal) level. The inset shows the proportion of individuals that became infected in *P. ramosa*-exposed treatments in each genotype (n = 12, each replicate consists of an individual *Daphnia*). Asterisks indicate if haemocyte numbers rose significantly (after sequential Bonferroni adjustment) above basal levels: ** $p < 0.01$, *** $p < 0.001$.

DISCUSSION

Just hours after exposure to the bacterial parasite *P. ramosa*, there was a large increase in the number of amoeboid cells circulating in the haemolymph of *D. magna.* These data also revealed very large differences in cellular response between host genotypes, ranging from no increase to a greater-than nine-fold increase in cell number (Figures 2.2, 2.4). Basal (uninduced) haemocyte counts did differ across host genotypes (Figure 2.3), but these differences did not predict the likelihood of becoming infected. This differs from the finding that *Drosophila melanogaster* with a greater basal haemocyte level were more resistant to parasitoid infection (KRAAIJEVELD *et al.* 2001). Non-infective parasite spores (*i.e.* those I heat-killed prior to exposure) elicited a small increase in the number of circulating haemocytes, suggesting that the presence of parasite material in the gut may trigger weak immune reactions; perhaps bacterial ligands are penetrating the gut mucosa and triggering an immune response (RAZ 2010)? However, data from the killed-spore experiment clearly show that live infective spores induce a much stronger cellular response.

This cellular response is possibly the host immune response that the parasite encounters when it passes from the host gut into its body, supporting very early work showing *D. magna* mounts a cellular response to a yeast-like infection (METCHNIKOFF 1884). Immune function and immunity, however, are clearly *not* one and the same: the largest increase in cell numbers was seen in the host genotypes that were susceptible to the parasite (Figure 2.4). Other studies of putative immune responses found no link between infection status and strength of the response (MUCKLOW *et al.* 2004). If the cellular immune response to *P. ramosa* depends upon the parasite spores passing the gut epithelium, complete resistance appears to be

achieved by preventing that passage (as opposed to destroying parasites once they have gained access). A very strong cellular response thus appears to be indicative of a critical failure elsewhere in the host immune system (most likely in the gut epithelium), and it appears that the gut epithelium forms the main defence.

The *P. ramosa* infection process may be similar to that seen in *Pasteuria penetrans,* a sterilising parasite that initiates infections by attaching to the heparin binding domain and gelatin binding domain proteins on the cuticle of *Meloidogyne* nematodes (MOHAN *et al.* 2001; SAYRE and STARR 1985; SCHMIDT *et al.* 2008). The external surface of the nematode encounters *P. penetrans* as it migrates through the soil, whereas *P. ramosa* is thought to be taken up as the *D. magna* filter feed where it then penetrates the gut. Aside from this difference in the location of infection, *P. ramosa* may similarly require binding to epithelial proteins to initiate infection, and without this binding the infection process, subsequent cellular response will not occur. The probability of molecular binding to *D. magna* epithelial proteins appears to be subject to host genetic variation; or, there is variation in other gut-based defences. I propose that a lack of molecular matching explains cases of resistance, while a strong cellular response indicates a molecular genetic match that allows parasites to overcome gut defences. This heuristic model of a two-tiered defence is largely supported by the observation that the three susceptible host genotypes had the strongest cellular responses, while the majority of non-responding genotypes remained healthy (Figure 2.4). Still, two host genotypes responded to parasite exposure but showed no signs of infection, which indicates that the cellular immune response may only play a limited role in resistance, if only a very small number of spores reach the haemolymph.

Previous work has modelled the genetics of infection as a two-stage process, with 'matching-allele' (MA) genetics for parasite detection, and 'gene-for-gene' (GFG) genetics for parasite eradication (AGRAWAL and LIVELY 2003). *Daphnia magna*'s patterns of resistance and cellular responses to *P. ramosa* can be used to test such models. Thus, a desirable follow-up study to the present work comparing host genotypes would be experiments incorporating both host and parasite genetic variation (CARIUS *et al.* 2001), as well as with parasites from other taxa, where a cellular response may successfully provide resistance. Studies of such genetic specificity and the cellular response would be the next step towards elucidating the immunological basis of invertebrate coevolutionary interactions.

A substantial body of work in invertebrate immunology has studied the response to opportunistic bacteria, generalist entomopathogens or chemical pathogen mimics (*e.g.* LPS); and there are considerable merits in measuring immune function in non-coevolved systems (BARNES and SIVA-JOTHY 2000), primarily that the parasite has not had the opportunity to evolve avoidance of host immune responses (BARNES and SIVA-JOTHY 2000; HUXHAM *et al.* 1988). By adopting such an approach one can better assess the generality of a host's immune function without the confounding influence of anti-parasite defence mechanisms. Conversely, my use of a naturally coevolving host-parasite combination means the cellular response I document reflects how invertebrates defend themselves against natural enemies. Indeed, outside of the well-studied interactions between mosquitoes and *Plasmodium* (JARAMILLO-GUITERREZ *et al.* 2009), and *Drosophila* and their parasitoids (FELLOWES and GODFRAY 2000), we have little understanding of the invertebrate immune response to coevolving biological enemies. Thus, in the study of

invertebrate immunity, my work is a rare example of the (putative) immune response, and genetic variation for that response, against a natural parasite.

It is now widely acknowledged that a stronger immune response does not necessarily lead to higher fitness – the relationship between host fitness and both size of immune response and parasite burden may not be linear (ADAMO 2004; STJERNMAN *et al.* 2008; VINEY *et al.* 2005). My work is a compelling example of this point: had I measured only haemocyte responsiveness without assessing infection probabilities (and hence fitness), a misleading impression of which is the fittest genotype would have emerged. This argues against the practice (common in the early days of ecological immunology) of measuring immune parameters in isolation from infection biology. Moreover, the large differences in cellular response between host genotypes emphasises the need to embrace genetic variation when studying immune function. Had I looked for a cellular response in just one host genotype, my results would very much depend on which genotype I studied. For example, a study of host genotype 3 would lead to opposite conclusions to a study of genotype 18. This makes clear the need to effectively link studies of immune function to studies of infection outcome in multiple host genotypes. That being so, the next stage is to investigate the role of initial parasite dose: perhaps large numbers of parasite spores are able to overcome host barrier defences, elicit a cellular response and cause infection in host genotypes found to be resistant in this study. We also need to explore the role of parasite genetic variation: both how it modifies cellular response in different host genotypes, and how this links to infection outcome.

CHAPTER 3

Parasite dose effects on immune activity and fitness in a *Daphnia*-microparasite system

There is often substantial variation in the number of infectious agents, *i.e.* the infective dose that immune systems encounter. This study examined how the number of circulating haemocytes (a measure of host immune activity), host fitness and parasite fitness were modified by initial parasite dose in a naturally coevolving host-parasite system: the crustacean *Daphnia magna* and its sterilizing bacterial parasite, *Pasteuria ramosa.* The magnitude of cellular response increased with the initial dose of parasite spores, as did the probability of infection in susceptible host genotypes; resistant hosts did not mount a cellular response, nor did they suffer any infection at high initial parasite doses. While I found density-dependent regulation of parasite reproduction (parasites had a lower fitness potential from infections that resulted at higher doses), host haemocyte number did not impact on parasite fitness potential; increased haemocyte number was associated with reduced host fitness.

INTRODUCTION

Parasites frequently cluster over both space and time, and the majority of parasites are carried by only a few hosts (ANDERSON and MAY 1991; EBERT *et al.* 2000). One consequence of this is variation in infective dose when host-parasite encounters occur. A host that encounters more parasite transmission stages is typically more likely to suffer infection (BEN-AMI *et al.* 2008b; BRUNNER *et al.* 2005; DE ROODE *et al.* 2007; OSNAS and LIVELY 2004) and thus a reduction in fitness (BEN-AMI *et al.* 2010; BRUNNER *et al.* 2005; TIMMS *et al.* 2001). Increased initial parasite dose also has an effect on parasite fitness following infection, which is sometimes negative (EBERT *et al.* 2000), and sometimes positive (DE ROODE *et al.* 2007). The host immune system frequently stands between successful and failed infection, however, while the relationship between initial parasite dose on the role and fitness is well studied, the role of immune activity in mediating this relationship is less well understood.

The relationship between dose and the immune response may not be straightforward: a strong host immune response can reflect an ability to resist parasitism and thus high host fitness; however, it can just as easily reflect host exposure to parasites and an impending fitness decline (FAULKNER *et al.* 2001; GRAHAM *et al.* 2011; VINEY *et al.* 2005). Further, the strength and efficacy of a particular host immune function may depend on the number of parasite transmission stages it has to defend against: an immune response is more likely to be elicited when the host is confronted with a high parasite dose, but immune defences that are effective when parasites are at low densities might be completely overwhelmed when faced with a high density of parasites.

The interaction between the crustacean *Daphnia magna* and its bacterial pathogen *P. ramosa* is well studied, and one striking pattern from this system is that different genotypes show dramatically different susceptibilities to the pathogen: for any particular *P. ramosa* strain, some host genotypes are highly susceptible, while others are seemingly completely resistant (AULD *et al.* 2010; CARIUS *et al.* 2001; LITTLE and EBERT 2001). In a recent study on 16 host genotypes, such phenotypic patterns were shown to be associated with a cellular immune response: a strong host cellular response (increase in circulating haemocytes) was generally associated with genotypes that went on to suffer infection (AULD *et al.* 2010). These findings suggested that resistance to *P. ramosa* may in most cases be due to a genetically determined barrier defence, perhaps in the gut (the probable site of initial penetration into the host), and that when this barrier is overcome, parasites enter the host and stimulate an immune response. Whether or not the cellular immune response reduces the cost of infection once it is established was not clear from these earlier studies. Moreover, the association between susceptibility and a cellular response was not entirely dichotomous: two of the 16 genotypes had smaller, but significant cellular responses and yet did not suffer infection. In these genotypes, immune response cells may be eliminating the parasites once they have passed the gut and are establishing an infection.

Thus, I hypothesised that host genotypes fall into three distinct categories of infection phenotype (Figure 3.1; adapted from (AULD *et al.* 2010). Some genotypes have an effective barrier mechanism, and therefore do not show either infection or a cellular response. I call these genotypes 'resistant' (Figure 3.1). Other genotypes lack the barrier defence and show both infection and a cellular response. I call these

genotypes ‘susceptible’ (Figure 3.1). A third group of genotypes lack the barrier defence and thus show a cellular response, but they nonetheless do not suffer infection, perhaps because a mechanism acting *via* the cellular response eliminate the bacterium as it attempts to establish itself. I call these genotypes ‘responders’ (Figure 3.1). I hypothesised that experimentally varying the exposure dose of parasites could shed light on the mechanisms of defence in this system. Specifically, I could a test whether the efficacy of the barrier mechanism really is all or nothing in nature, or whether high parasite dose leads to infections in host genotypes that have hitherto been assigned to the ‘resistant’ category. Similarly, increasing dose may reveal that ‘responders’, which ordinarily kill parasites with haemolymph-based immune defences at low doses, are not able to do so at higher doses. I used six of the genotypes from Figure 3.1: two ‘susceptible’ (GG4 and GG17), two ‘resistant’ (GG16 and GG18), and the two ‘responders’ (GG20 and GG22).

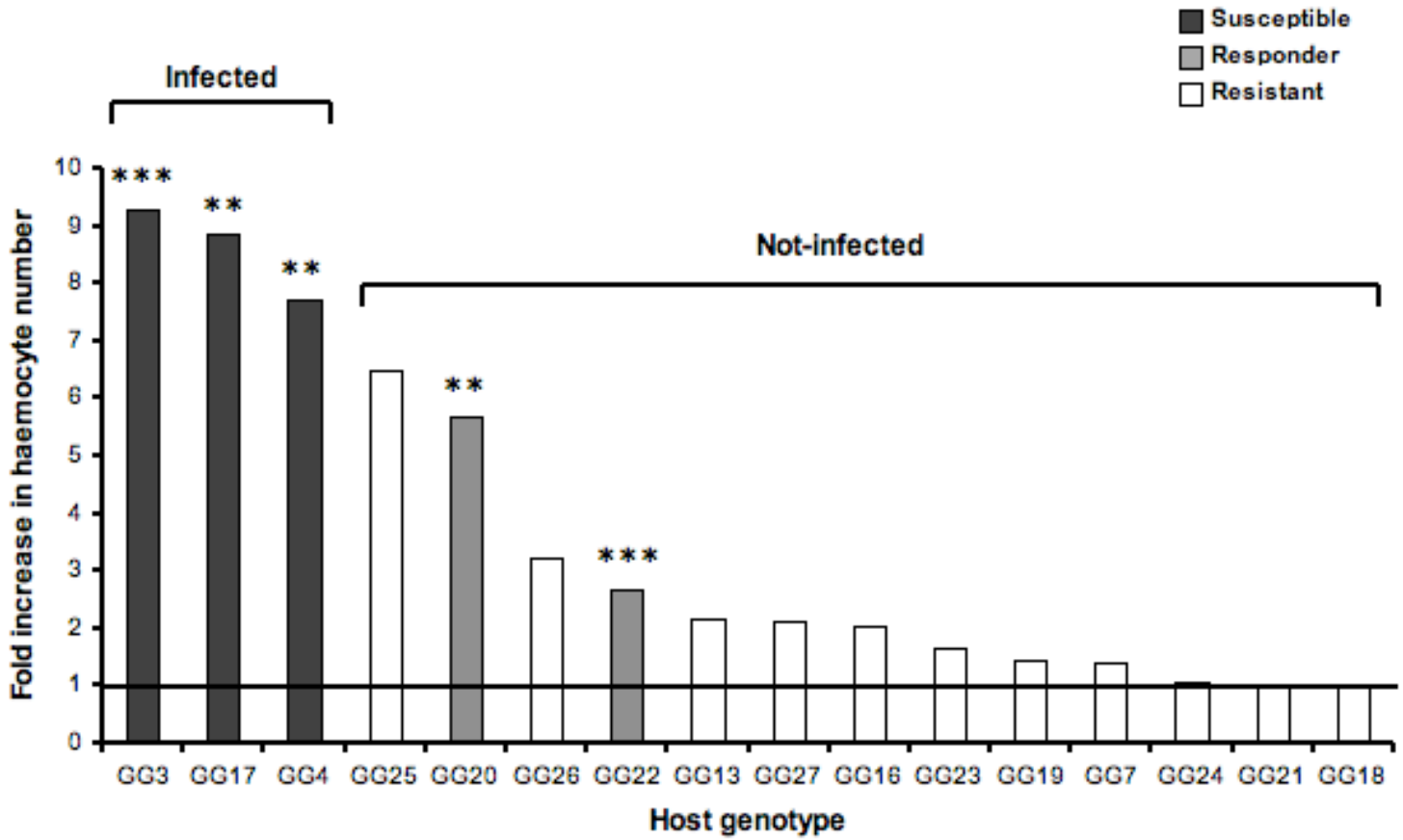

Figure 3.1. Magnitude of host cellular response (measured as fold induction of haemocyte numbers following exposure to *P. ramosa*). Asterisks indicate if haemocyte numbers rose significantly above basal levels (after sequential Bonferroni adjustment): ** $p < 0.01$, *** $p < 0.001$.

MATERIALS AND METHODS

Study organisms

Daphnia magna are crustaceans that inhabit still freshwater pools, and like most organisms, *Daphnia* are host to a variety of parasites, including fungi, microsporidia and bacteria (EBERT 2008; GREEN 1974). This study uses the bacterial endoparasite, *Pasteuria ramosa*. *Daphnia* ingest *P. ramosa* transmission spores when filter-feeding and successful infection occurs when the spores pass from the host's gut to its haemolymph. The parasite then sequesters host resources for its own development and reproduction; resources that would be otherwise used by the host to reproduce (EBERT *et al.* 2004). This renders the host completely sterile (and thus genetically dead EBERT *et al.* 1996). On the death of the host, mature spores are released into the environment; thus, *P. ramosa* transmission is exclusively horizontal (EBERT *et al.* 1996). The diagnosis of infection is relatively straightforward, due to *Daphnia's* clear carapace: *P. ramosa*-infected hosts cease reproduction, lack eggs or developed ovaries and have a red-brown bacterial growth in their haemolymph.

Daphnia magna reproduce by cyclical parthenogenesis; they reproduce asexually in the main, but have sex when environmental conditions become unfavourable (CARVALHO and HUGHES 1983; HOBAEK and LARSSON 1990; SLARSARCZYK *et al.* 2005). By keeping *Daphnia* in favourable conditions in the laboratory, it is possible to maintain clonal lines, allowing complete control over host genotype.

Experimental design

The six host genotypes and the parasite isolate used in this study were from a pond near Gaarzerfeld in Germany. Five of the six host genotypes (GG16, GG17, GG18, GG20 and GG22) were founded from single *Daphnia,* each hatched from a resting egg (ephippium) in the laboratory in 2009 (see AULD *et al.* 2010), and maintained clonally since then. These ephippia were from sediment samples collected in 1997. The sixth genotype (GG4) was founded from a single adult *Daphnia* (also collected in 1997), and has since been maintained in a state of clonal reproduction (CARIUS *et al.* 2001). The *P. ramosa* isolate (Sp.1) originated from a single infected *Daphnia* from the Gaazerfeld pond (CARIUS *et al.* 2001), and the spore suspension used here consisted of five Sp1-infected GG4 *Daphnia* homogenised in 6 ml of ddH_2O.

This experiment had six parasite dosage treatments, including a control (0, 10, 100, 1000, 10000 and 1.0×10^6 spores per replicate), and there were 8-12 replicates per treatment. A replicate consisted of five *Daphnia* in 200 ml of artificial medium (KLUTTGEN *et al.* 1994) with one twentieth of the prescribed SeO_2 concentration (EBERT *et al.* 1998). All jars were incubated at 20°C on a 12:12 light/dark cycle and fed 1.0 ABS chemostat-grown *Chlorella vulgaris* algal cells, where ABS refers to the optical absorbance of 650 nm white light by the *C. vulgaris* culture. Medium was refreshed three times per week, and after the *Daphnia* had a clutch of offspring. Replicate jars were kept for three generations to minimise phenotypic variation due to the environment or maternal effects. Throughout all stages of the experiment, the location of replicate jars within trays, and the location of trays within the incubator were randomized in order to remove any positional effects.

Second clutch *Daphnia* offspring from the third generation were used for experimentation. These day-old offspring were all grouped according to replicate, and then split across the twelve experimental treatments, analogous to a split-brood design. Each experimental replicate consisted of five *Daphnia* neonates in 200 ml artificial medium, and replicates were kept in the same conditions as the previous two generations until at least three of the five *Daphnia* deposited eggs in their brood pouch, at which point they were exposed to parasite treatment.

The number of circulating haemocytes was chosen as a measure of host immune activity. Haemocytes are central to the invertebrate innate immune system, as they are directly involved in phagocytosis and encapsulation (ATAEV and COUSTAU 1999; PECH and STRAND 1996; ROTH and KURTZ 2009), and are vehicles for other important immune effectors, such as reactive species of oxygen and nitrogen, phenoloxidase and antimicrobial peptides (JOHANSSON and SODERHALL 1985; MITTA 2000; STRAND 2008). A cellular response was detected in *D. magna* as early as 1884 (METCHNIKOFF 1884), and *Daphnia* are known to mount a cellular response in the presence of *P. ramosa* (AULD *et al.* 2010).

Parasite treatment and host haemocyte counts

On the day of parasite treatment, the spore isolate was gently thawed, thoroughly vortexed and concentration of *P. ramosa* spores was calculated from counts obtained with a Neubauer counting chamber (0.0025 mm^2 × 0.1 mm depth) under 40 × magnification. Experimental replicates were placed in the well of a 24-well cell plate (Costar, Corning Inc., NY), with 1 ml of artificial medium. The designated dose of *P. ramosa* spores was then added to the well, and thoroughly mixed with the

medium using a pipette, and control replicates received a placebo of 10 μl of homogenised healthy *Daphnia* solution (which consisted of four uninfected *Daphnia* homogenised in 6 ml of ddH_2O). The duration of *P. ramosa* exposure was five hours, since previous work showed that this genotype of *Daphnia* mounts a cellular response to *P. ramosa* five hours after exposure to the parasite (AULD *et al.* 2010).

After the parasite treatment period, the five *Daphnia* from each replicate were washed in fresh artificial medium. Four of these *Daphnia* were dried on a paper towel, placed on a glass Petri dish and had their hearts pierced with a 25-gauge needle (BD Microlance, Drogheda, Ireland). A 2 μl pipette was then used to extract 0.5 μl of haemolymph from each *Daphnia,* and this haemolymph was mixed thoroughly with 4 μl of anticoagulant buffer (98 mM NaOH, 186 mM NaCl, 17 mM EDTA and 41 mM citric acid, pH adjusted to 4.5: LAVINE *et al.* 2005), giving a total of 6 μl of haemolymph-buffer solution. This solution was mixed thoroughly with a pipette and 2 μl was placed in a fertility counting chamber [0.001 mm^2 x 0.100 mm (depth)] (Hawksley, Lancing, Sussex, UK), and the density of amoeboid haemocytes was determined. The fifth *Daphnia* was placed in a jar with 60 ml of artificial medium for life history assays.

Host and parasite fitness assays

Daphnia were fed 1.0 ABS of *C. vulgaris* cells per day, and their medium was changed three times per week and after the host had a clutch of offspring. Jars were checked daily for offspring production, and the number of offspring was recorded with each clutch, and then removed. The total offspring count was my principal

measure of host fitness. From day 25 days post-parasite exposure, hosts were checked for evidence of *P. ramosa* infection.

The experiment was terminated after 35 days, when surviving *Daphnia* were individually frozen at -20°C in 1.5 ml Eppendorf tubes. The number of mature transmission spores per *Daphnia* (a measure of parasite fitness) was later determined from these frozen samples. To obtain spore counts, hosts were homogenized in 100 µl of ddH_2O with a sterile pestle, and the number of spores was determined using a Neubauer (Improved) counting chamber (0.0025 mm^2 × 0.1 mm depth).

Data analyses

All analyses were performed using R (IHAKA and GENTLEMAN 1996; R 2005). First, I tested whether host genotype (GG4, GG16, GG17, GG18, GG20 and GG22) and the initial dose of parasite spores influenced the number of circulating haemocytes using analysis of covariance, where initial parasite dose was treated as a continuous variable and host genotype was treated as a fixed factor. Inspection of plots of the data suggested a possible nonlinear relationship between haemocyte number and initial parasite dose, so I included both Log_{10}[dose] and $(Log_{10}[dose])^2$, and all two-way interactions. I also analysed host offspring counts using a general linear model and the same explanatory variables. Haemocyte numbers could not be included as an explanatory variable in this model, because it was too closely associated with dose. So, as a secondary analysis, I examined how host offspring counts were associated with the number of circulating haemocytes in all hosts and then in infected hosts only using Spearman rank correlations.

Finally, I studied parasite fitness in terms numbers of *P. ramosa* transmission spores per infected host. Since only host GG4 suffered infections at all parasite doses, analysis was restricted to this genotype. I used a general linear model with Log_{10}[haemocyte number], Log_{10}[dose], $(Log_{10}[dose])^2$ and all two-way interactions included as explanatory variables. In all cases, model reduction was performed using a stepwise backwards procedure (CRAWLEY 2007).

RESULTS

Haemocyte counts were obtained from 1592 *Daphnia* from 398 jars. It was clear that exposure to *P. ramosa* resulted in a cellular response in some host genotypes but not others (Table 3.1). Specifically, haemocyte number increased substantially with initial parasite dose and levelled off at high doses in the two 'susceptible' genotypes (GG4 and GG17), and exhibited a smaller linear increase with initial parasite dose in one of the 'responder' genotypes (GG20; Figure 3.2).

Infection with *P. ramosa* only occurred in genotypes that mounted a cellular response (GG4, GG17 and GG20 Figure 3.3). Infection reduced host reproductive success substantially: infected hosts had 34.41 ± 0.94 offspring, whereas their healthy counterparts had 77.75 ± 1.93 offspring. Thus, for offspring production, there was a strong host genotype by dose interaction driven by the fact that only some genotypes suffered infection (Figure 3.4, Table 3.2). Similarly, haemocyte number was negatively associated with host reproduction (r_s= - 0.19, $p < 0.001$), because haemocyte number was elevated only in susceptible hosts. In infected hosts only, there haemocyte number was not associated with host reproduction (r_s = -0.26, $p = 0.11$) .

A total of 39 hosts went on to suffer infection after exposure to *P. ramosa* (of which 30 were GG4 hosts), and the number of transmission spores per infected host varied from 250,000 to 10,950,000. The final number of transmission spore counts exhibited a negative relationship with initial parasite dose in GG4 hosts (Table 3.3; Figure 3.5).

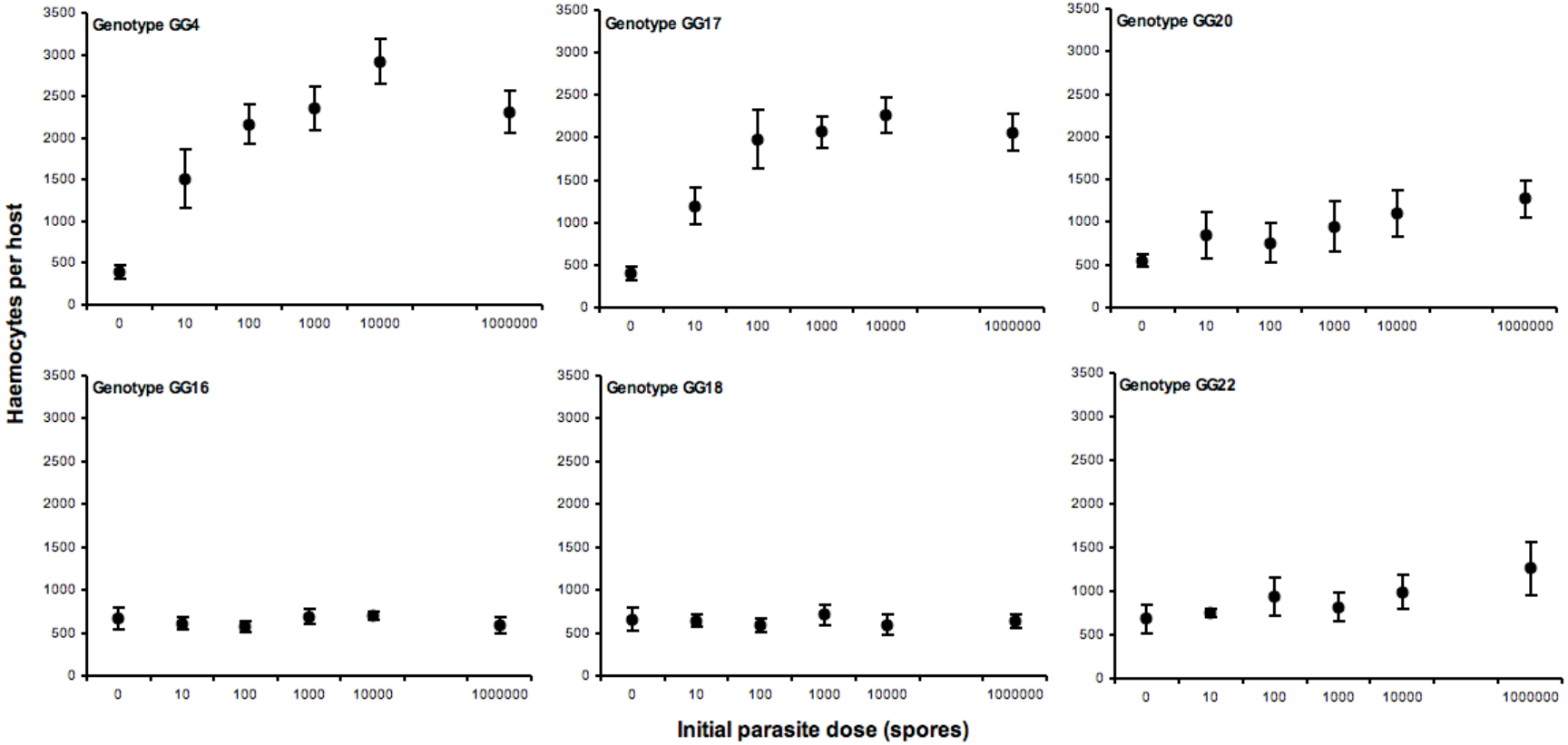

Figure 3.2. Haemocyte number in relation to initial parasite spore dose in all six host genotypes.

Table 3.1. Summary of statistical analysis testing the effects of initial parasite dose and host genotype on the number of circulating haemocytes.

	DF	Type III SS	*F*	*p*
Haemocyte number (all hosts)				
Log_{10}[dose]	1	31522875	74.90	**< 0.0001**
$(Log_{10}[dose])^2$	1	17233351	40.95	**< 0.0001**
Host genotype	5	964802	0.46	0.81
Log_{10}[dose] x Host genotype	5	29709900	14.12	**< 0.0001**
$(Log_{10}[dose])^2$ x Host genotype	5	18110345	8.61	**< 0.0001**
Error	380	159925285		

Table 3.2. Summary of statistical analysis testing the effects of initial parasite dose, the number of circulating haemocytes and host genotype within host category on host lifetime reproductive success.

	DF	Type III SS	*F*	*p*
Host offspring number				
Log_{10}[dose]	1	5654	16.57	**< 0.0001**
Host genotype	5	7217	4.23	**< 0.001**
Log_{10}[dose] x Host genotype	5	6599	3.87	**< 0.01**
Error	386	131648		

Table 3.3. Summary of statistical analysis testing the effects of initial parasite dose and the number of circulating haemocytes on the number of parasite transmission spores per infected GG$ host 30 days after host exposure to the parasite.

	DF	Type III SS	*F*	*p*
Parasite spore number (infected GG4 hosts)				
Log_{10}[haemocytes]	1	1.93×10^{12}	0.66	0.42
Log_{10}[dose]	1	1.27×10^{12}	4.34	**< 0.05**
Error	26	7.58×10^{13}		

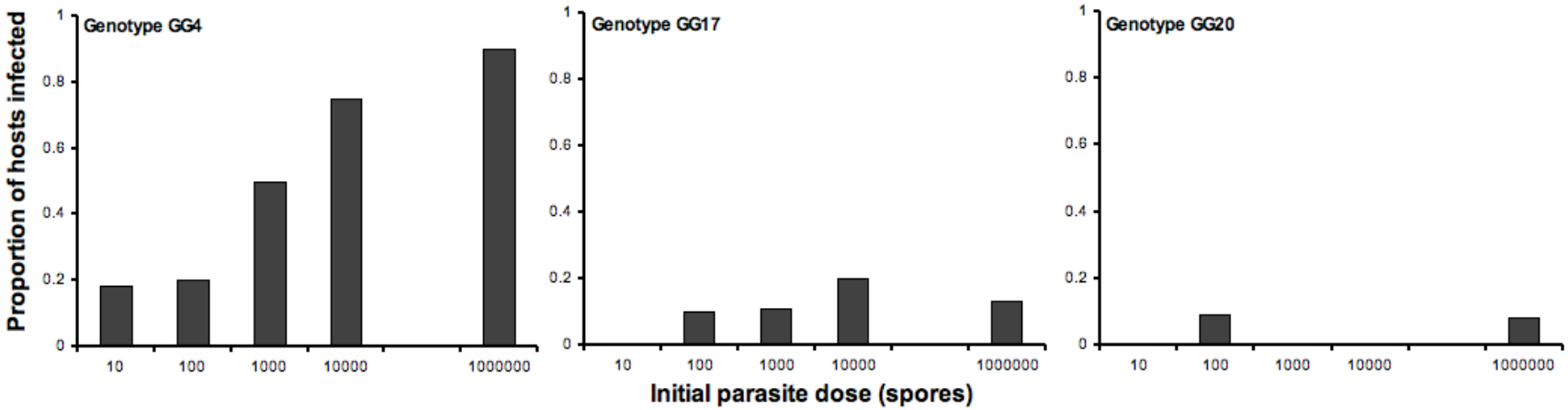

Figure 3.3. The proportion of infected hosts in relation to initial parasite spore dose in host genotypes GG4, GG17 and GG20. The three genotypes (GG16, GG18 and GG22) not represented here suffered no infections.

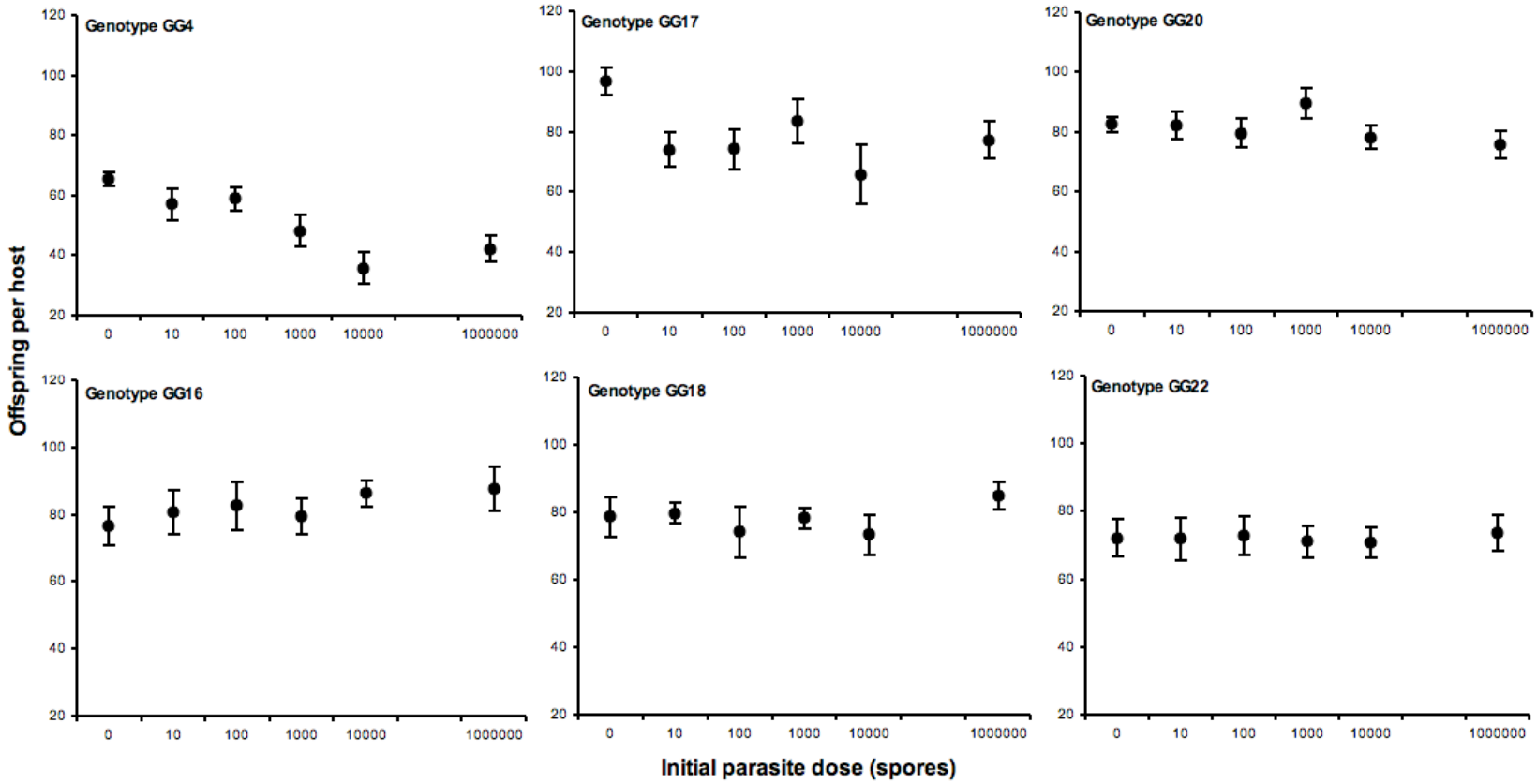

Figure 3.4. Host offspring production in relation to initial parasite spore dose in all six host genotypes.

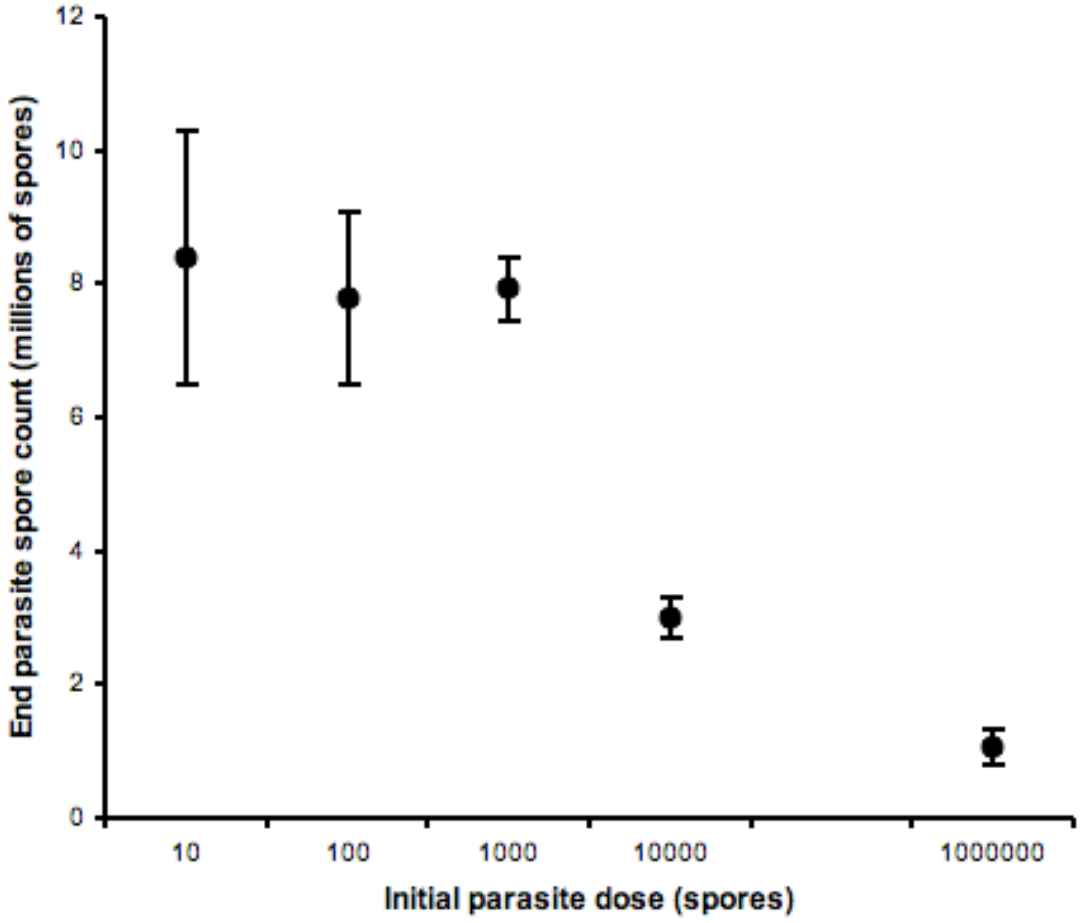

Figure 3.5. The number of parasite transmission spores per infected GG4 host relating to initial parasite dose.

DISCUSSION

Daphnia magna mounted a cellular response to *P. ramosa,* but only in genotypes where infection was observed (GG4, GG17 and GG20). Amongst genotypes that showed at least some susceptibility, haemocyte number increased with the initial dose of parasite spores (Figure 3.2). The cellular response was strongest in GG4, which suffered the most *P. ramosa* infections, and weakest in GG20, which suffered the fewest (Figure 3.1). The plateau in haemocyte numbers at higher parasite doses may be due to the hosts reaching their maximal cellular response in these host genotypes.

A central aim of this study was to examine whether any relationship between dose and either host or parasite fitness were mediated by haemocyte number. Again focusing mainly on genotypes that showed at least some susceptibility, increasing dose lead to lower host fitness (Figure 3.4). Haemocyte numbers increased with exposure to parasites, and then further with increasing dose (Figure 3.2). The *Daphnia* cellular response is thus an accurate reporter of infection and subsequent host fitness losses. To some degree haemocyte number is also a reporter for parasite fitness, simply because haemocyte counts are higher in infected hosts (and parasites only achieve fitness when they infect). However, as dose (and haemocyte numbers) increase, parasite fitness goes down (Figure 3.5). Thus at higher doses, haemocyte numbers, because they remain relatively high, do not report that parasite fitness has declined. While it could be that the host cellular response is only effective at combating parasites when the initial dose is high (hence the decline in parasite fitness at high dose), it seems more plausible that this decline in parasite fitness is density-dependent reproductive success, as has been

observed in both micro- and macro-parasites (BEGON *et al.* 1990), including *P. ramosa* (EBERT *et al.* 2000).

Together, these data suggest that haemocytes do not play a role in preventing infection or limiting parasite replication once infection has occurred. However, haemocytes are also known to be important for would-healing in a number of invertebrates (see THEOPOLD *et al.* 2004). The *Daphnia* cellular response might therefore provide a fitness benefit by repairing damage caused by the infecting *P. ramosa* spores. If this were so, one would expect infected hosts with the strongest cellular responses to have the highest fitness in the face of parasitism. These data provide no evidence for this: there is no association between the magnitude of cellular response and host offspring production.

My findings support the two-stage heuristic model for infection outlined previously (AULD *et al.* 2010), where stage one involves the parasite passing from the host's gut to its haemolymph (eliciting a cellular response) and stage two involves overcoming host haemolymph-based immunological effectors (resulting in successful infection). In our previous study, both GG20 and GG22 mounted weak cellular responses, but neither genotype suffered any *P. ramosa* infections, suggesting that the cellular response, in some cases, caused the elimination of parasites that had successfully crossed the gut boundary (AULD *et al.* 2010). Here, GG20 mounted a cellular response and suffered two infections, whereas GG22 did not mount a cellular response and suffered no infection. Also, haemocyte numbers in GG20 exhibited a weak linear relationship with parasite dose, suggesting that most of the *P. ramosa* spores are being halted at the gut epithelium, but that a small number of spores were reaching the host's haemolymph and eliciting a cellular response. Data from GG22 also

support this hypothesis: although there was no significant cellular response in GG22, there was a trend towards increased haemocyte number following parasite exposure (Figure 3.1), suggesting that a very small number of *P. ramosa* spores were able to reach the host's haemolymph.

The role of an immune system is to protect its owner against the fitness-reducing effects of parasitism (FRANK 2002). This often leads to the assumption that hosts with highest immune activity are the most immune to parasites, and therefore have the highest fitness. My findings emphasize how flawed this assumption can be. In this naturally coevolving host-parasite system, an elevated number of circulating haemocytes is positively associated with future parasitism, probably because haemocyte number is an indicator of how may parasite spores pass from the host gut into the haemolymph. It is, however, important to note that infection in the *Daphnia–Pasteuria,* system is dependent on the specific combination of host and parasite genotypes (CARIUS *et al.* 2001), and *Daphnia* infection with other parasite species is also dependent on the specific combination of *Daphnia* genotype and parasite species (DECAESTECKER *et al.* 2003). I can therefore not exclude the possibility the *Daphnia* haemocytes may be effective against different genotypes of *P. ramosa,* or different parasite species. In any case, my results provide compelling support for the argument that measures of host immune activity must be linked to both host and parasite fitness (ADAMO 2004; GRAHAM *et al.* 2011; VINEY *et al.* 2005), and that such studies should be conducted using naturally occurring host-parasite systems in order to draw biologically relevant conclusions regarding host immunocompetence (LITTLE *et al.* 2005a).

CHAPTER 4

Associations between host cellular response and genetic specificity for infection in a *Daphnia*-microparasite system

The probability of infection (or the severity of infection once it is established) is often dependent on the specific pairing of host and parasite genotypes; this is termed genetic specificity. Our understanding of the mechanistic basis for this phenomenon is, however, lacking. This study examined the link between host cellular response (the proliferation of circulating haemocytes following parasite exposure) and infection outcome in a naturally coevolving host-parasite system: the crustacean, *Daphnia magna* and its sterilizing bacterial parasite, *Pasteuria ramosa*. Genetic specificity clearly governed the probability of infection, and a cellular response only occurred in host-parasite genotype combinations where infection occurred. These data suggest host susceptibility is mainly determined by the parasite's ability to penetrate host barrier defences and that the immune response is merely a marker for infection. These data also support the argument that increased immune activity does not always indicate high fitness: here, the most immune responsive hosts were sterilized and thus had no future fitness.

INTRODUCTION

For invertebrate hosts, the probability of becoming infected following exposure to a parasite is often dependent on the specific pairing of host and parasite genotypes; there is an effect above and beyond the additive contributions of individual host and parasite genomes. For example, it is common that no single host genotype can exclude all parasite genotypes and no single parasite genotype can infect all host genotypes. This is genetic specificity, and it manifests statistically as a host genotype-by-parasite genotype ($G_H \times G_P$) interaction (LAMBRECHTS 2010; SCHMID-HEMPEL and EBERT 2003). Genetic specificity has profound implications for the coevolution of hosts and their parasites. First, it indicates a mechanism for the maintenance of polymorphism (BYERS 2005), as each parasite can only select against a subset of the host population and *vice versa.* Second, genetic specificity can foster negative frequency dependent selection (where being a rare genotype is advantageous) (HAMILTON 1980; JAENIKE 1978). A recent meta-analysis of 500 studies of animal and plant hosts has shown genetic specificity to be widespread (WILFERT and SCHMID-HEMPEL 2008), but little is known of its mechanistic basis.

The outcome of host-parasite interactions may often depend on the host immune system. Genetic specificity has been documented in invertebrate-parasite models (CARIUS *et al.* 2001; SCHMID-HEMPEL and REBER 2004), and yet invertebrate immune systems are thought to only distinguish between broad classes of infectious agents (Gram positive bacteria, Gram negative bacteria, fungi *etc.*) (FERRANDON *et al.* 2003; LEMAITRE *et al.* 1997). Also, invertebrate immune effectors are believed to be cross-protective: insect antimicrobial peptides have been shown to be responsive (and

effective) against a number of very different microorganisms (TZOU *et al.* 2002), including some that have never coevolved with the host (CASTEELS *et al.* 1994). However, whilst molecular immunologists have discussed the generality of invertebrate immune pathways, evolutionary ecologists have been discovering genetic specificity, as described above, through the study of whole organism phenotypes (CARIUS *et al.* 2001; LITTLE *et al.* 2005a; SCHMID-HEMPEL and REBER 2004). There is, however, a distinct lack of studies addressing the role that immune responses might play in the phenomena of genetic specificity (but see (RIDDELL *et al.* 2009).

This study examines whether any link between host immune function and the infection outcome (and thus host and parasite fitness) is dependent on the specific pairing of host and parasite genotypes. Specifically, whether a $G_H \times G_P$ interaction for immune efficacy explains genetic specificity for the probability of a host suffering infection, or the fitness consequences of infection for host or parasite or both antagonists. I used a naturally coevolving host-parasite system, the crustacean *Daphnia magna* and its sterilizing bacterial parasite *Pasteuria ramosa,* where genetic specificity is well described and quite substantial (CARIUS *et al.* 2001). The number of circulating haemocytes was chosen as the measure of host immune activity because haemocytes are known to be important anti-parasite defences in a number of invertebrates (ATAEV and COUSTAU 1999; ELROD-ERICKSON *et al.* 2000; KRAAIJEVELD *et al.* 2001; PHAM *et al.* 2007), either by phagocytosing or encapsulating invading parasites, or as vehicles for other humoral immune effectors (NAPPI and OTTAVIANI 2000; STRAND 2008).

Early research has shown *Daphnia magna* maintain circulating amoeboid haemocytes (METCHNIKOFF 1884), and that some *Daphnia* genotypes mount a rapid cellular response to *P. ramosa*; *i.e.,* the number of circulating haemocytes is massively

increased (AULD *et al.* 2010). In a comparison of 16 genotypes, it was shown that the *Daphnia* with the strongest cellular response to *P. ramosa* are *most* likely to get infected, and all non-responding *Daphnia* remained uninfected. These findings led to the formulation of a two-stage heuristic model for infection in this system: stage one involves the parasite successfully entering the host, evidenced by the presence of a cellular response; and, stage two involves the parasite successfully overcoming the host's immune defences (AULD *et al.* 2010). However, Auld *et al* (2010) studied just one parasite strain.

Here, I examine *Daphnia* cellular responses under both host and parasite genetic variation, replicating parasite exposure across multiple host-parasite genetic combinations, some of which result in infection and some of which do not. I then tested whether genotype specificity for infection rested on host cellular immunity, *i.e.,* whether a host's cellular response led to host resistance against some parasite genotypes but not others, or if genetic specificity for infection depended on whether the parasite overcame host barrier mechanisms, in which case the efficacy of host cellular response would not rest on a $G_H \times G_P$ interaction. Finally, I examined the fitness consequences of the host cellular response in terms of both host lifetime reproduction and parasite within-host population growth.

MATERIALS AND METHODS

The host-parasite model

Daphnia magna Straus is a small crustacean that inhabits still freshwater pools. *Daphnia magna* are cyclically parthenogenetic: they reproduce mainly by ameiotic

parthenogenesis, but are sexual under specific conditions, typically, short photoperiod, food stress or crowding (KLEIVEN *et al.* 1992). By keeping *Daphnia* in good conditions, it is possible to maintain them clonally, and thus to replicate and maintain independent clonal lineages. This allows the dissection of the relative contributions of environment and genotype on a particular *Daphnia* phenotype.

Pasteuria ramosa is an obligate bacterial endoparasite of *Daphnia*, and its transmission spores are ingested when the host is filter feeding. Once inside the gut, these spores infect the *Daphnia,* and undergo development in the host's haemolymph. In order to develop and reproduce, *P. ramosa* sequesters resources that would otherwise be used by the host for its own reproduction, resulting in host sterilization (EBERT *et al.* 1996). The next generation of parasite transmission spores are released on the death of the host, and transmission is exclusively horizontal (EBERT *et al.* 1996).

Host genotypes and parasite isolates

This study used six *Daphnia* genotypes (named GG4, GG16, GG17, GG18, GG23 and GG26) and five *P. ramosa* isolates (named Sp1, Sp8, Sp13, Sp17, Sp23). Hosts GG4 and parasites Sp1 and Sp8 were originally collected from a pond in Gaarzerfeld, Germany in 1997 and were used in a previous study showing that the probability of infection is dependent on the specific combination of host genotypes and parasite isolates (CARIUS *et al.* 2001). These two host genotypes have since been kept in a state of clonal reproduction, and the parasite spores were frozen at -20°C. Hosts GG16, GG17, GG18, GG23 and GG26 originated from resting eggs (ephippia) in sediment collected from the same population at the same time as the other hosts, though I did not

hatch them until 2009. Ephippia were washed in ddH_2O, hatched in the laboratory, and one individual from each was clonally propagated.

Parasite isolates Sp17 and Sp23 were obtained by exposing hosts to sediment collected from Gaarzerfeld; a single infected host was then randomly chosen and homogenised in 5 ml of ddH_2O to make a spore suspension. The name of the parasite isolate indicates the host genotype it originated from: Sp17 is from infected host GG17 and Sp23 is from infected GG23. These spore isolates were propagated by re-exposing them to healthy *Daphnia* (of the same original host genotype), giving rise to further infections.

Experimental design

Ten replicates of each clonal genotype were kept for three generations to minimise variation in maternal effects. A replicate consisted of three jars, each containing five *Daphnia* and 200 ml of artificial *Daphnia* medium (KLUTTGEN *et al.* 1994). *Daphnia* were fed 1 ABS of chemostat-grown *Chlorella vulgaris* algal cells per *Daphnia* per day, (ABS refers to optical absorbance of 650 nm white light by the *C. vulgaris* culture), and all jars were incubated at 20°C on a 12:12 hour light/dark cycle. Medium was refreshed three times per week, or after the *Daphnia* had a clutch of offspring.

I took the offspring from each maternal replicate, dividing 30 offspring evenly into six treatment groups (*i.e.* five hosts per group): one for each of the five parasite isolates plus a control. Thus, a total of 1800 *Daphnia* were used in the experiment. These were kept in the same conditions as the maternal generations until three of the

five *Daphnia* deposited eggs in their brood pouch, at which point the replicate was ready for parasite exposure.

Parasite exposure and host cellular response

Before parasite treatment was administered, spore isolates were thawed, vortexed and thoroughly mixed using a pipette. The density of spores was determined using a Neubauer counting chamber (0.0025 $mm^2 \times 0.1$ mm depth) under 40× magnification. The *Daphnia* from each replicate were then placed in a well of a 24-well plate (Costar, Corning Inc., NY) with 1 ml of artificial medium. They received 100 μl (500 000 spores) of their designated parasite isolate, or 100 μl of homogenised healthy *Daphnia* if the replicate was a control.

After five hours of exposure to the parasite, *Daphnia* were washed in artificial medium. Four of the *Daphnia* from each jar were dried on a paper towel, placed on a Petri dish and their hearts were pierced with a 25 gauge needle (BD Microlance, Drogheda, Ireland). From each of the four *Daphnia,* 1.0 μl of haemolymph was pipetted and mixed with 4 μl of anticoagulant buffer (98 mM NaOH, 186 mM NaCl, 17 mM EDTA and 41 mM citric acid, pH adjusted to 4.5: LAVINE *et al.* 2005), giving a total of 8 μl of haemolymph solution. The haemolymph solution was mixed thoroughly with a pipette, and 2 μl was placed in a fertility counting chamber [0.001 $mm^2 \times 0.100$ mm (depth)] (Hawksley, Lancing, Sussex, UK), and the number of circulating haemocytes per *Daphnia* was counted. The fifth *Daphnia* from each replicate did not contribute haemolymph; it was placed singly in a small jar with 60 ml of artificial medium in order to study infection outcome and host and parasite fitness.

Infection outcome, host fitness and parasite fitness

Individual hosts were checked daily to see if they had reproduced. If they had a clutch, the age of reproduction (in days) and the number of offspring was recorded. Dead hosts were removed from the jars and their day of death recorded; they were then frozen in 1.5 ml eppendorfs at -20°C. On day 25 post-treatment, hosts were assessed for evidence of infection with *P. ramosa.* Symptoms of infection include a cessation of reproduction, absence of developed ovaries and bacterial growth in the haemolymph. In infected hosts, I also documented the day when reproduction ceased. Throughout the experiment, jars were kept in trays of 24, and the positions of both the tray in the incubator and jars within tray were randomised daily.

The experiment was terminated on day 40, when all surviving hosts were placed in a 1.5 ml eppendorf and frozen at -20°C. Counts of *P. ramosa* transmission spores in each infected host were determined as follows: individual *Daphnia* were homogenized with 100 μl of ddH_2O, and two independent counts were made from the resulting suspension in a Neubauer (Improved) counting chamber (0.0025 mm^2 × 0.1 mm depth) under 40× magnification.

Analysis of probability of infection and host haemocyte number

I first studied whether the probability of infection was determined by the specific host and parasite combinations used (*i.e.* I tested for genetic specificity). For this, the proportion of hosts that became infected was analysed by fitting a GLM with quasibinomial error distribution to data from parasite-exposed replicates only, with host genotype, parasite genotype, and their interaction included as fixed explanatory factors.

I initially studied haemocyte counts in a similar manner by fitting an ANOVA to counts from only parasite-exposed hosts, again fitting host genotype, parasite genotype, and their interaction as fixed factors.

As expected from this study population (CARIUS *et al.* 2001), the proportion of hosts becoming infected was highly dependent on the host and parasite combination, and two categories were clearly evident: infective host-parasite combinations, and non infective. Thus, I studied haemocyte counts by making a new explanatory variable with three biologically obvious categories: host-parasite combinations that never resulted in infection ('Non-infective combinations'), host-parasite combinations where at least infection was observed ('Infective combinations') and control hosts that were not exposed to parasites ('Controls'). I fitted a one-way ANOVA to test for variation in haemocyte counts across these groups, and used a Tukey's HSD test to look for differences between pairs of groups.

Analysis of host and parasite reproduction

Next, I tested for host life-history changes, in terms of offspring production, by fitting an ANOVA to offspring counts from only parasite-exposed hosts. Host genotype, parasite genotype, the number of circulating haemocytes and all interactions were included as explanatory variables. Infection will explain most of the variation in host reproduction, however, I postulated that exposure to parasites could also drive variation in reproduction in uninfected hosts. In some cases, the parasite may have passed from the host's gut to the haemolymph, and be subject to an immune response which could be potentially costly to the host, whereas in other cases, the parasite may have failed to

infect the host and will therefore not elicit a costly immune response. Thus, I analysed offspring counts in uninfected hosts by fitting a one-way ANOVA with host genotype nested within exposure category ('Control, 'Non-infective' or 'Infective'). Finally, using a general linear model, I analysed parasite transmission spore counts (from infected hosts only), again with host genotype, parasite genotype, the number of circulating haemocytes and all interactions included as explanatory variables. All analyses were performed using R (IHAKA and GENTLEMAN 1996; R 2005), and in cases where these data were non-orthogonal, type III (adjusted) sums of squares were used.

RESULTS

The proportion of hosts that became infected ranged from 0 to 70% depending on host genotype, parasite genotype and an interaction between the two (Table 4.1). I confirmed the well-established decline in host reproduction due to infection by comparing offspring counts of infected hosts to those of uninfected hosts with a *t*-test: infected hosts had significantly fewer offspring than their uninfected counterparts (30.57 ± 1.42 as opposed to 83.92 ± 114: $t = 29.27$, d.f. = 71.00, $p < 0.0001$).

Of the 36 host-parasite genotype combinations, there were 6 'Controls', 9 'Infective' combinations and 21 'Non-infective' combinations (Figure 4.1). Haemocyte counts significantly differed between these groups ($F_{2, 334} = 107.03$, $p < 0.0001$), and the highest counts were from the 'Infective' group (Table 4.1; Figure 4.2). There were no significant differences between haemocyte counts from the 'Control' and 'Non-infective' groups. Thus, there was only a cellular response in host-parasite combinations where successful infection was possible.

In parasite-exposed *Daphnia,* host reproductive success depended on host genotype ($F_{5,293}$ = 18.63, $p < 0.0001$) and the number of circulating haemocytes (r = -0.25, $F_{1,\ 293}$ = 19.43, $p < 0.0001$). In uninfected *Daphnia,* host reproductive success depended on infection group ($F_{2,\ 296}$ = 4.92, $p < 0.01$) and host genotype within infection group ($F_{13,\ 296}$ = 12.31, $p < 0.0001$). In particular, healthy *Daphnia* from the 'Infective' group had the most offspring, while offspring counts from the 'Non-infective' and 'Control' groups did not differ from each other (Figure 4.3).

Mortality during the experiment meant that only a fraction of infected hosts survived until day 40 (and mortality did not depend on treatment group), and thus spore counts were made from 24 infected hosts. The number of transmission spores from infected hosts was highly variable (between 5,000 and 11,745,000 spores), and depended on both host genotype and parasite genotype (Table 4.1; Figure 4.4); however, spore counts did not show any relationship the number of circulating host haemocytes (Table 4.1).

Table 4.1. Summary of statistical analyses showing the effects of host genotype and parasite genotype on the probability of successful infection and on the number of circulating haemocytes in hosts; and host reproduction, number of host haemocytes, host genotype and parasite genotype on the number of parasite transmission spores on day 40. All analyses were performed on data from parasite-exposed hosts only.

	d.f.	*F*	*P*
Infectivity			
Host genotype	5	26.17	**< 0.0001**
Parasite genotype	4	14.78	**< 0.0001**
Host x Parasite	20	4.60	**< 0.0001**
Error	299		
Haemocyte counts			
Host genotype	5	20.78	**< 0.0001**
Parasite genotype	4	31.92	**<0.0001**
Host x Parasite	20	8.67	**< 0.0001**
Error	278		
Parasite spore counts			
Host genotype	3	15.29	**<0.0001**
Parasite genotype	4	11.57	**<0.001**
Error	16		

	GG4	GG16	GG17	GG18	GG23	GG26	Mean
Sp1	**0.60**	0.00	**0.10**	0.00	0.00	0.00	0.12
Sp8	0.00	**0.10**	0.00	0.00	**0.20**	0.00	0.05
Sp13	0.00	0.00	0.00	0.00	**0.10**	0.00	0.02
Sp17	**0.70**	0.00	**0.20**	0.00	**0.40**	0.00	0.22
Sp23	0.00	0.00	0.00	0.00	**0.40**	0.00	0.07
Mean	0.26	0.02	0.06	0.00	0.22	0.00	0.10

Figure 4.1. The proportion of hosts infected for each host-parasite combination. Host genotypes are listed in columns and parasite genotypes are listed in rows. Host-parasite genotype combinations that experienced higher infectivity are darker. See Table 4.1 for statistical details.

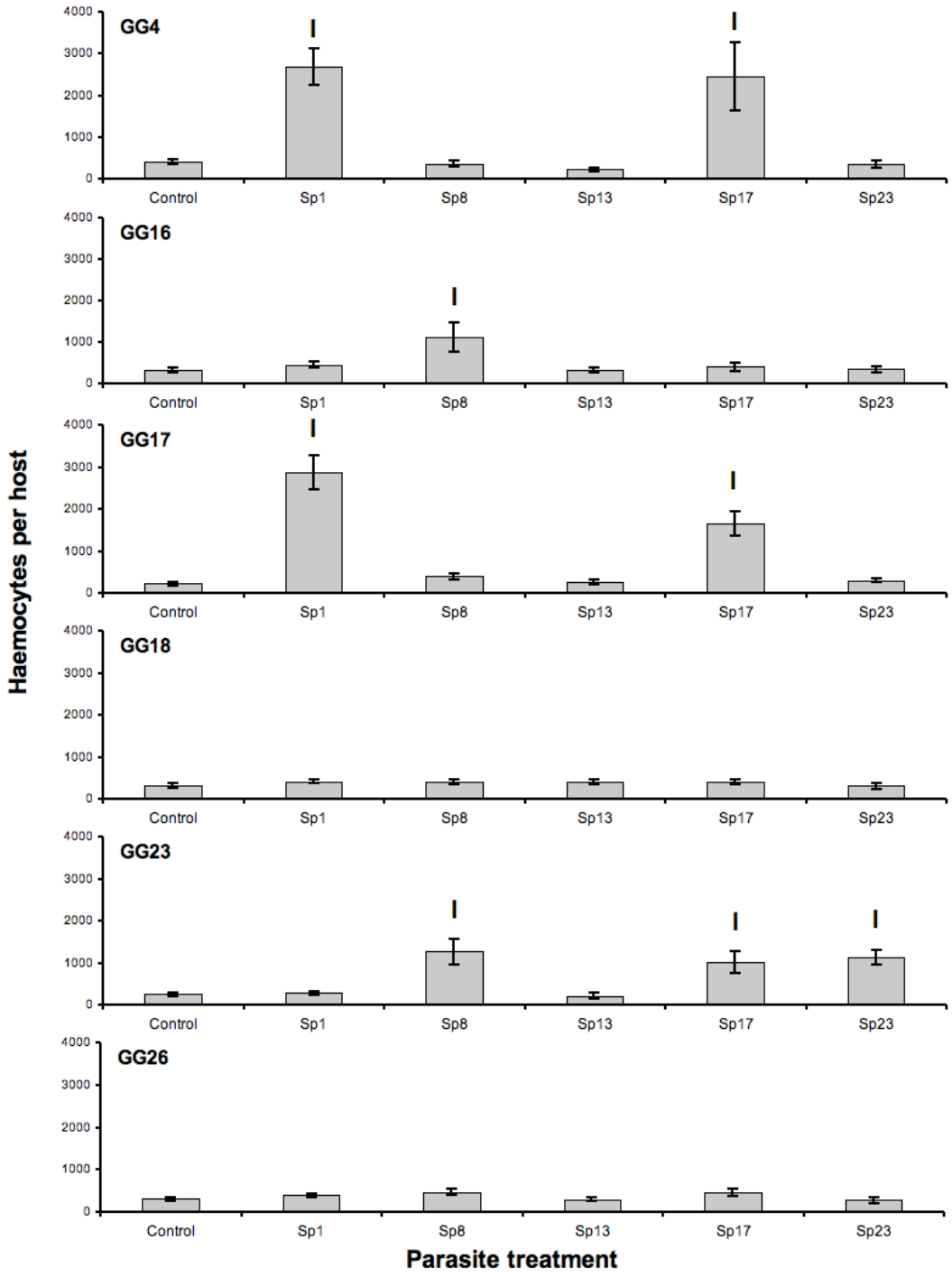

Figure 4.2. Haemocyte counts (± 1 S.E.) and infection outcome for multiple host-parasite genotypic combinations. Host-parasite combinations that resulted in infections are denoted with I.

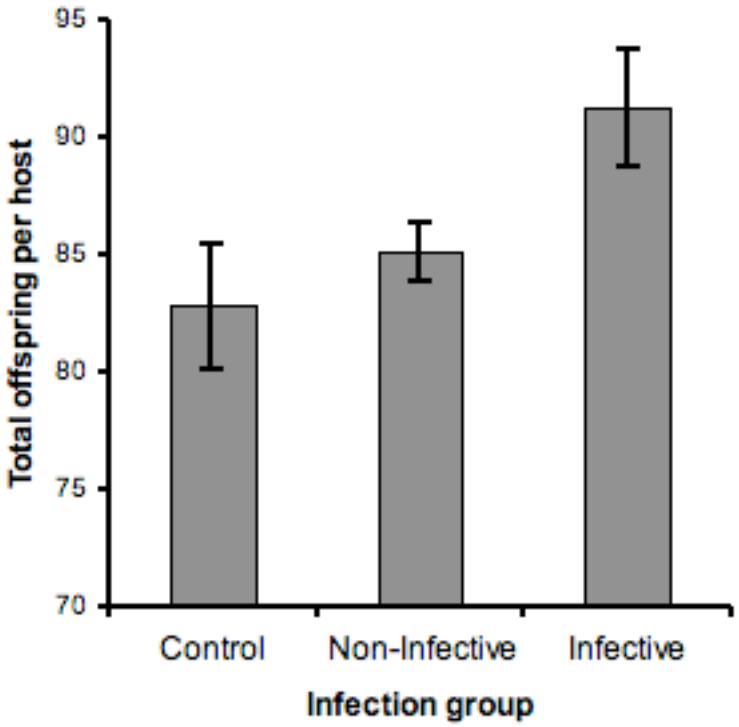

Figure 4.3. Offspring counts from healthy Daphnia (± 1 S.E.) from three groups of host-parasite combinations: 'Control', where the hosts were not exposed to parasite spores; 'Non-infective', where *Daphnia* were exposed to parasite spores, but parasitism never resulted; and 'Infective', where hosts were exposed to parasite spores that had the potential to result in parasitism.

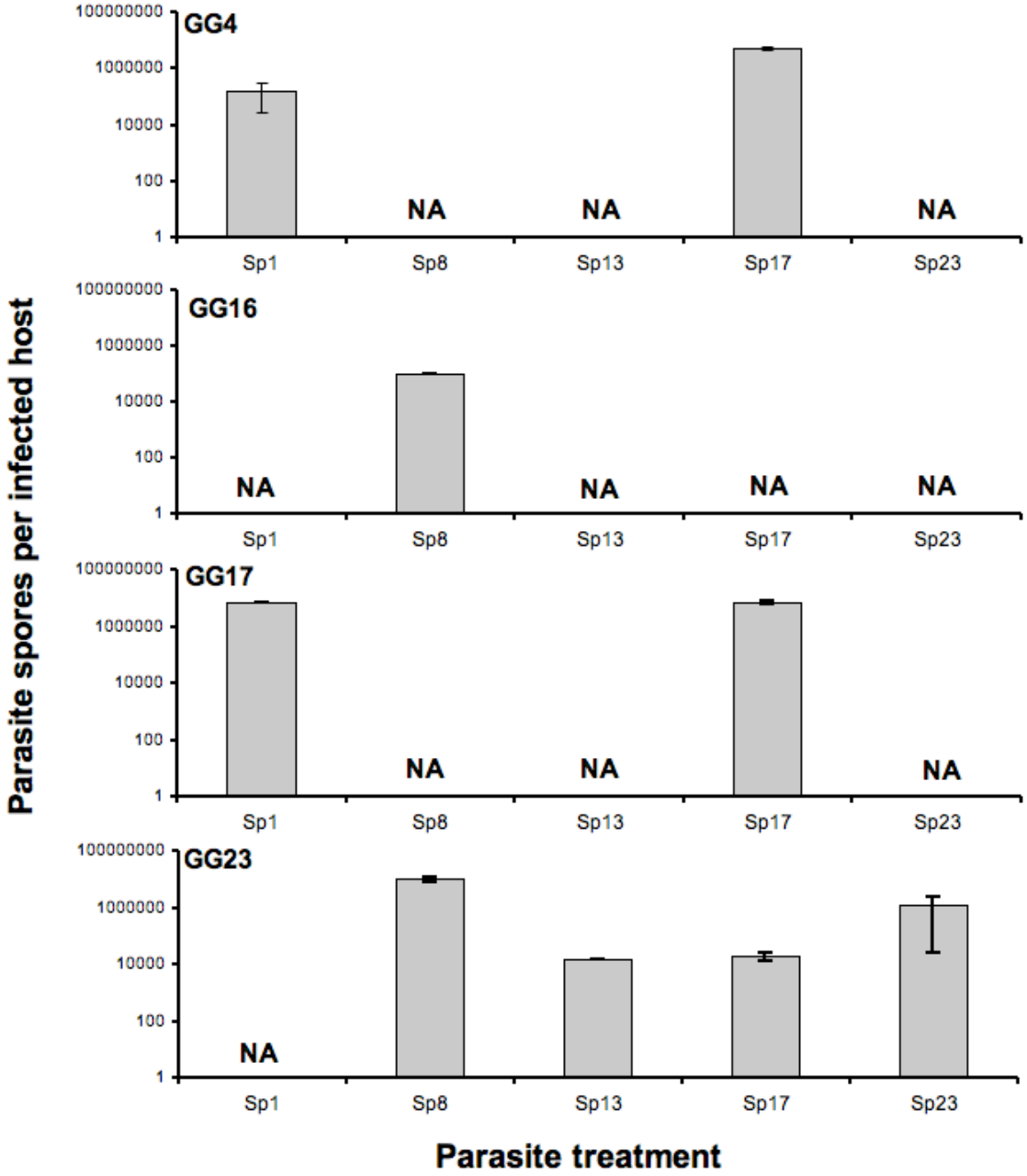

Figure 4.4. *Pasteuria ramosa* spore counts (± 1 S.E.) for multiple host-parasite genotypic combinations. Host-parasite combinations that did not result in infections are denoted with NA.

DISCUSSION

I examined how host haemocyte number associated with infection outcome and both host and parasite fitness in a system renowned for strong genetic specificity (Figure 4.1, but see also CARIUS *et al.* 2001; VALE and LITTLE 2009). I found that *Daphnia magna* mounts a strong cellular response against its sterilizing parasite *Pasteuria ramosa*, but only in host-parasite genotype combinations where infection occurred (Figure 4.2). Thus, the presence of a host cellular response is associated with susceptibility. Since *P. ramosa* is a sterilizing parasite, a strong cellular response predicts host genetic death and thus a termination of host fitness.

Previous work has modelled infection from the perspective of host resistance, where hosts must first detect the parasites and then eliminate them (AGRAWAL and LIVELY 2003), and a more recent model has approached infection from a parasite's perspective, where the parasite has to actively recognise the host (FENTON *et al.* 2009). Findings from this study support a two-stage heuristic model that incorporates both host and parasite contributions to infection: the onus is on the parasite to get into the host, but if it does, it is subject to the host's immune system. (AULD *et al.* 2010). The two-stage model posits that the occurrence of a cellular response indicates that the parasite has overcome host barrier defences, and has passed from the gut to the haemolymph (stage one); and a strong cellular response suggests the parasite is subject to an array of haemolymph-based immune defences in the host and is likely to reproduce and achieve fitness (stage two). The data from this study are consistent with this model. Host reproductive success depended on the identity of the host genotype and the number of circulating haemocytes; however, the absence of a host genotype-by-parasite genotype

interaction suggests that host reproduction is not affected by the identity of the parasite *per se*, rather just the parasite's ability to pass from the host's gut to the haemolymph (where a cellular response is elicited).

Hosts often pay a fitness cost for successful defence against parasites (HASU *et al.* 2006; LITTLE and KILLICK 2007), or in response to immune stimulation from an artificial elicitor (MORET and SCHMID-HEMPEL 2000), though this does not seem to be a prevalent phenomenon in *Daphnia* (LABBÉ *et al.* in press). Hosts also sometimes alter their reproductive investment upon exposure to biological enemies. For example, fecundity compensation (where hosts that are exposed to infective parasites shift their reproductive effort earlier in their lives) has been documented in a number of different organisms (BLAIR and WEBSTER 2007; PAGAN *et al.* 2008; SCHWANZ 2008), including *Daphnia magna* (in response to exposure to a microsporidian parasite: (CHADWICK and LITTLE 2005) and to *P. ramosa* (EBERT *et al.* 2004). So, there are two different predictions for parasite-exposed but uninfected hosts: one from a cost of immunity perspective, one from a fecundity compensation perspective. Here, I found evidence for fecundity compensation in *Daphnia magna* when exposed to *P. ramosa.* Specifically, among hosts that were exposed to the parasites but remained healthy, those from 'Infective' host-parasite combinations had the most offspring (Figure 4.3). The fact this only occurred in 'Infective' combinations is unsurprising, given that there is no evidence that *P. ramosa* can infect (and thus alter host life history) in 'Non-infective' combinations. What is more, strength of this fecundity compensation depended on the identity of the host genotype, meaning it could be subject to parasite-mediated natural selection.

Finally, whilst the numbers of transmission spores per infected host were variable (Figure 4.4), I found no relationship between the number of circulating host haemocytes and the final number of parasite transmission spores in infected hosts (Table 4.1). Therefore, whilst increased haemocyte number is a predictor of likely infection, and therefore a good predictor of whether or not the parasite will achieve fitness, it does not associate with within-host parasite growth, and is therefore not predictive of the magnitude of parasite fitness. Thus, as far as the parasite is concerned, the main hurdle to achieving fitness is successfully passing from the host gut to the haemolymph (see also Chapter 3).

Infection may not occur because of two reasons: either the parasite does not get past the host's barrier defences, or it is eliminated by haemolymph-based immunological mechanisms. From the parasite's perspective, these two outcomes could be very different in terms of fitness consequences. If the parasite overcomes host barrier defences, but is then destroyed by host haemolymph-based immune effectors, it is dead. Conversely, if the parasite fails to penetrate the host's barrier defences, it may not be killed; it may have the opportunity to infect a different host in the future and achieve fitness at a later time. Thus, failed infection may not always mean zero parasite fitness. A desirable follow-up study to the present work would be to compare infection outcomes in hosts that were exposed to the faeces of other hosts that had been exposed to either 'Infective' or 'Non-infective' parasite spores. The results of such a study will strengthen our understanding of the fitness consequences of unsuccessful infections (KING *et al.* in press).

This study provides robust support for the argument that stronger immune responses *do not* always mean elevated immunity and increased host fitness (ADAMO

2004; GRAHAM *et al.* 2011; VINEY *et al.* 2005). Had I used haemocyte number as a proxy for host resistance, I would have falsely concluded that the hosts with the strongest cellular response would be the fittest. In actuality, the exact opposite is the case: a rapid proliferation in the number of circulating host haemocytes is an effective predictor of successful parasitic infection and future host sterilization, and, this is true under both host and parasite genetic variation. This study also highlights the importance of barrier defences (in this case, the gut epithelium). Indeed, the presence of a cellular response indicates a failure of host barrier defences, and in many cases this failure is catastrophic, as infection-induced sterilization causes host genetic death.

CHAPTER 5

Elevated haemocyte number is associated with infection and low fitness potential in wild *Daphnia magna*

Immune activity may be a cause of resistance to parasites, but it can also be a consequence of infection. Thus, the functional significance of an immune response is more accurately assessed when it is measured alongside both host fitness and infection status. However, surprisingly few ecoimmunology studies have achieved this, and many assume greater immune activity equals greater host fitness. I sought to determine the significance of immune responses in a naturally coevolving host-parasite system in the wild, with support from laboratory experiments. I measured haemocyte numbers in *Daphnia magna* in relation to an infection that has a clear fitness consequence: infection with the bacterium *Pasteuria ramosa* causes sterilization. Haemocyte number was consistently elevated in infected *Daphnia* in the field, and in parasite exposed or infected hosts in the laboratory. These results provide a clear example where increased immune activity does not mean increased immunity or fitness. Indeed, haemocytes apparently are not a cause of resistance- they are a symptom of infection, and because *P. ramosa* sterilises hosts, *Daphnia* with the highest haemocyte counts have extremely low health.

INTRODUCTION

Parasites reduce the fitness of the hosts they infect, and their ubiquity makes them an important selective agent for many organisms. Host immune systems, by preventing parasite establishment and/or proliferation, may consequently play an important role in preserving fitness of infected organisms. But what exactly is the relationship between immune response magnitude and fitness? In some cases, the intuitive scenario will apply: a stronger immune response will be helpful, and will lead to greater host fitness by reducing the harm caused by infection. However, strong immune responses have drawbacks as well, as they can drain energy reserves or cause immunopathology, thus reducing host fitness. Moreover, greater immune activity could equally reflect a greater current parasite burden (and hence susceptibility), or past exposure (and thus a successful defence). In other words, an immune response can be either a cause or a consequence of parasite burden (GRAHAM *et al.* 2011; OSNAS and LIVELY 2006). Thus the relationship between immune response magnitude and fitness may be complex, and more immune responsiveness will not always mean more fitness (DAY *et al.* 2007; GRAHAM *et al.* 2005; GRAHAM *et al.* 2011).

These complications highlight that drawing conclusions about the role of host immunity in parasite-mediated selection requires the simultaneous measurement of three parameters: immune activity, host fitness and infection status (BRADLEY and JACKSON 2008; GRAHAM *et al.* 2005; GRAHAM *et al.* 2011; VINEY *et al.* 2005), preferably across genetic and environmental variation (LAZZARO and LITTLE 2009), and/or in the field with natural parasites (STASZEWSKI *et al.* 2007). Much pure immunological work is poorly poised to achieve this, as the careful work required to

elucidate immunological mechanism requires tightly controlled conditions, inbred strains and often the use of artificial stimulants of the immune response.

Ecological immunology (ecoimmunology) has sought to fill this gap, but the proportion of these studies that measure all three parameters of immune activity, infection status, and host fitness is relatively small. To highlight this point, I searched Web of Science for literature on ecoimmunology (precise search criteria are described in methods) and sorted the results into four groups depending on whether they measured: (1) immune activity only; (2) immune activity and infection status (*e.g.* either quantification of parasite burden, or simply whether an individual is infected or not); (3) immune activity and a measure of host fitness; (4) all three. I found that 42% of recovered studies only measured host immune activity, 15% measured host immune activity and infection status, 27% measured host immune activity and subsequent host fitness, and only 16% measured all three traits. It is thus clear that a large fraction of ecoimmunology studies can only assume what more immune activity actually means for hosts. The assumption that more immune activity means greater fitness has long been criticised (ADAMO 2004; BEHNKE *et al.* 1992; GRAHAM *et al.* 2011; READ and ALLEN 2000; SADD and SCHMID-HEMPEL 2009; VINEY *et al.* 2005), but my survey suggests that it remains prevalent: the 42% of studies that only incorporated immunological measurements included many that were published since 2006 and for which assumptions about the immune response-fitness relationship have never been tested. The current study is a empirical example of how heightened immune activity can be associated with low, not high, host fitness.

Using the crustacean *Daphnia magna* and its naturally coevolving parasite, *Pasteuria ramosa* as a model, I measured, in the field, infection status and host immune

activity. Because *P. ramosa* sterilises its host, infection status simultaneously provides a measure of host fitness. In *Daphnia,* I counted circulating host haemocytes as a measure of immune activity, because haemocytes generally play an important anti-parasite role in invertebrates (ATAEV and COUSTAU 1999; CANESI *et al.* 2002; COTTER *et al.* 2004; ELROD-ERICKSON *et al.* 2000; KRAAIJEVELD *et al.* 2001). A very early study showed *Daphnia* maintain a population of circulating amoeboid haemocytes (METCHNIKOFF 1884), and my more recent work has shown that some genotypes of *Daphnia* mount a cellular response shortly after exposure to *P. ramosa* (AULD *et al.* 2010).

As part of my field survey, I recorded both haemocyte number and *P. ramosa* prevalence in a natural *D. magna* population over a nine-month period, during which several epidemics were documented. I complemented these observations with two laboratory experiments subjecting *Daphnia* from this same population to controlled exposure to *P. ramosa* spores (also collected from the same local population). First, I tested for an early cellular response in parasite-exposed hosts (five hours post-exposure); second, I tested whether well-established infection was associated with longer-term differences in haemocyte number (21 days post-exposure). Thus, I was able to determine if any association between wild host immune activity, infection status, and fitness was mirrored under controlled laboratory conditions.

MATERIALS AND METHODS

Host and parasite organisms

Daphnia magna is a cyclically parthenogenetic freshwater crustacean that lives in shallow eutrophic ponds. They are host to the obligate microparasite, *Pasteuria ramosa*: a spore-forming bacterium that is transmitted horizontally from the corpses of previously infected hosts (EBERT *et al.* 1996). Infection occurs when *Daphnia* filter-feed, taking in the transmission spores along with their food. Once in the host, the *P. ramosa* spores go through a 10-20 day developmental process, resulting in many millions of transmission spores that are released on the death of the host. Infection nearly always results in the complete sterilization of the host.

Field collections and haemocyte counts

Daphnia magna were sampled from a pond at Kaimes Farm, Leitholm, Scottish Borders (2˚20.43'W, 55˚42.15'N) twice per month between April and October 2009, and once in November and December. Adult *Daphnia* were grouped according to infection status (healthy or infected; infection can be easily diagnosed by eye, as the symptoms of include an absence of eggs in the brood pouch or a lack of enlarged ovaries, redness and obvious bacterial growth in the haemolymph). Hosts from each grouping were placed five at a time in a cell extraction chamber with 4.0 μl of ice-cold anticoagulant buffer (98 mM NaOH, 186 mM NaCl, 17 mM EDTA and 41 mM citric acid, pH adjusted to 4.5: LAVINE *et al.* 2005), and their hearts were pierced using a 25-gauge needle (BD Microlance, Drogheda, Ireland), causing haemolymph to pool into the buffer. This

haemolymph-buffer solution was then transferred into a 0.5 ml Eppendorf tube and placed on ice for the hour-long journey back to the laboratory. In the laboratory, each of the samples was mixed thoroughly using a pipette, and 2 μl were placed in a fertility counting chamber [0.001 mm^2 × 0.100 mm (depth)] (Hawksley, Lancing, Sussex, UK), and the number of haemocytes was counted. These counts were converted to number of cells per microlitre of haemolymph-buffer solution.

I also measured a set of variables within the pond at each sampling date: water temperature was measured using a digital field thermometer (HANNA instruments HI93510), and the population density of *Daphnia* was estimated by sweeping a 0.063m^2 net through one metre of pond water from three fixed locations around the pond. These live collections were taken back to the laboratory, and counts of infected adults, healthy adults and juveniles were determined. The population density (in *Daphnia*/litre) of each life stage was then just this count divided by the volume of water the net was passed through (water volume = 0.063 m^2 (net area) × 1m (the sweep distance)). *Pasteuria ramosa* infection was assessed in the adult portion of all subsamples: this was usually done by eye, but in the few ambiguous cases, individuals were crushed under a glass coverslip on a microscope slide, and then examined under a transmission microscope for the presence of *P. ramosa* spores.

Experimental setup

First, I tested whether exposure to *P. ramosa* resulted in a rapid increase in haemocyte count in *Daphnia*, as seen previously in a laboratory study of a different population of *D. magna* (AULD *et al.* 2010). This first experiment is referred to below as the early

cellular response experiment. Second, I exposed hosts to parasites but then waited (21 days) for infections to become established, and then tested whether *Daphnia* with established infections had greater haemocyte counts than their exposed but uninfected counterparts. This scenario more closely resembles the hosts I collect from the wild, which have established infections. This second experiment is referred to below as the infection experiment. In both experiments, *Daphnia* were exposed to a mixture of *P. ramosa* isolates, as opposed to a single isolate, in order to more accurately mimic *Daphnia-Pasteuria* interactions in the field (wild *Daphnia* will likely encounter spores from different genotypes).

Methods were similar to Auld *et al.* (2010). For both experiments, independent replicates of four *Daphnia* genotypes (KA25, KA30, KA71 and KA93) were kept in the laboratory in a state of clonal reproduction for three generations, to minimize variation in condition. Hosts were kept in groups of five in jars containing 200 ml of artificial medium (KLUTTGEN *et al.* 1994) and fed 5.0 ABS of chemostat-grown *Chlorella vulgaris* algal cells per day (ABS is the optical absorbance of 650 nm white light by the *C. vulgaris* culture). Their medium was changed three times per week and jars were incubated at 20°C on a 12L:12D light cycle. Second clutch neonates formed the experimental replicates in each of the two experiments.

The parasite spores used here were from a solution containing a mix of *P. ramosa* isolates from the Kaimes pond (ALLEN and LITTLE in press). This spore solution was prepared by homogenising multiple *P. ramosa* -infected hosts with ddH_2O, yielding a solution containing a mixture of spore isolates.

Early cellular response experiment

Replicates were allocated to one of two parasite treatments: parasite-exposed or non-exposed (control). There were six replicates per parasite treatment, per genotype. Experimental replicates were kept in the same conditions as maternal generations until at least three of the five *Daphnia* deposited eggs in their brood pouch, at which point they were ready for parasite exposure. Parasite treatments were carried out as follows: for each replicate, the five adult *Daphnia* were placed in the well of a 24-well plate (Costar, Corning Inc., NY, USA). The parasite spore solution was thawed, thoroughly mixed with a pipette and the number of spores was determined using a Neubauer (Improved) counting chamber (0.0025 mm^2 × 0.1 mm depth). Replicates assigned to the parasite-exposed treatment then received 100 000 *P. ramosa* spores, and control replicates received an identical volume of homogenised healthy *Daphnia* as a placebo.

Treatment exposure lasted for five hours, after which the *Daphnia* were removed from the cell plate and washed in artificial medium. Hosts from each replicate were placed in a cell extraction chamber, their hearts were then pierced and their haemolymph pooled in 4 μl of ice-cold anticoagulant buffer. Haemocytes were then counted using methodology described earlier. These *Daphnia* were exposed to the parasite as adults to ensure I obtained enough haemolymph from which haemocyte numbers could be estimated reliably.

Infection experiment

There were 12 parasite-exposed replicates and six control replicates per genotype, with five *Daphnia* per replicate. Parasite exposures were carried out as follows: for each

replicate, the five female neonates (< 24 hours old) were placed in a jar with 200 ml of artificial medium and 5 g of sterile sand. The parasite spore solution was thawed, mixed and the spores were counted as before. Parasite-exposed replicates received a dose of 100 000 *P. ramosa* spores; control replicates received the same volume of placebo. Jars were stirred daily and fed low amounts (1.5 ABS per day) of *C. vulgaris* throughout the infection period, which lasted 7 days. The low food levels forced the *Daphnia* to filter-feed the sand at the bottom of the jars. This procedure was meant to mimic a natural infection process, where *Daphnia* ingest spores from the sediment.

On day 8, the replicates were changed into clean jars with 200 ml of fresh artificial medium, and they were then fed 5.0 ABS of *C. vulgaris* per day and their medium was refreshed three times per week. On day 21, hosts from each replicate were grouped according to infection status and placed in the cell extraction chamber with 0.8 µl of ice-cold anticoagulant buffer per host. The hosts' haemolymph was extracted and their haemocytes were counted using the methodology described earlier.

Analysis

All data were analysed using general linear models in R (IHAKA and GENTLEMAN 1996; R 2005), and model simplification was performed by removing the highest order non-significant term and examining the explanatory power of subsequent models (CRAWLEY 2007). The heterogeneity of variance was assessed for all models and the assumptions for the tests were fulfilled, and the model fitting process was repeated until a minimum adequate model was achieved.

Field data

I aimed to test whether haemocyte numbers in the field could be explained by infection status (infected or not), host density, parasite prevalence or temperature. However, I considered that the haemocyte numbers could reflect current conditions, or might more closely reflect events occurring in either the past or the future. I therefore examined how current haemocyte counts were influenced by current infection status, parasite prevalence (current, past or future), host density (current, past or future) and temperature (current and past). I reasoned that it was unlikely for future pond temperature to affect current haemocyte counts, and so did not include future temperature in my models.

All models included sample date (when haemocyte counts were made) as a random effect, to control for any temporal autocorrelation. The fixed effects in each model were as follows:

$$H_t = I_t + \text{Prev}_t + \text{Dens}_t + \text{Temp}_t + \varepsilon$$

$$H_t = I_t + \text{Prev}_{t-2} + \text{Dens}_{t-2} + \text{Temp}_{t-2} + \varepsilon$$

$$H_t = I_t + \text{Prev}_{t+2} + \text{Dens}_{t+2} + \varepsilon$$

Where H is the haemocyte count, I is the infection status (infected or healthy), 'Prev' is the population parasite prevalence, 'Dens' is the host population density, 'Temp' is the pond temperature and ε is the error. The subscript t denotes the lag (in weeks) between when haemocyte counts were made and the other variables were recorded. Since the all the variables were determined from hosts that were removed from the pond, the data fall into discrete populations, and are thus independent over time. I therefore used GLMs with quasipoisson error structure to analyse my data.

As a final analysis of the field data, I hypothesised that high parasite prevalence might be followed by a reduction in host population density. This hypothesis was tested by examining whether there was a correlation between current (arcsine square root transformed) parasite prevalence and host density two and four weeks in the future.

Experimental data

Data from the cellular response experiment were used to test whether controlled exposure to *P. ramosa* led to an increase in the number of circulating haemocytes, and whether any response differs between the four host genotypes (*i.e.* if there was a *P. ramosa*-exposure-by-genotype interaction for haemocyte number).

Data from *P. ramosa*-exposed hosts from the infection experiment were used to test whether long-term parasitic infection, host genotype or an interaction between these two factors affected the number of circulating haemocytes. The infection experiment data were non-orthogonal in nature, so I therefore fitted my models using adjusted sums of squares (type III sums of squares). Finally, I examined whether host genotype affected the proportion of parasite-exposed hosts that became infected with *P. ramosa*, using a generalized linear model (GLM) with quasibinomial error structure. The significance of host genotype as an independent variable was determined by removing it from the original model (leaving the null model), and analysing the resulting change in deviance (CRAWLEY 2007).

Ecoimmunology literature search

I performed a Web of Science literature search to determine the proportion of ecoimmunological studies that measured (1) immune activity only; (2) immune activity and infection status; (3) immune activity and a measure of host fitness; (4) all three traits. I used the search term "ecoimmunology OR eco-immunology OR ecological immunology" on the 21st of September 2010, and discarded studies that were reviews, technical reports, or theoretical studies, as well as those that did not measure a host immune response. Infection status was either a quantification of parasite burden or simply a record of whether the host was infected or not. Host fitness was typically as a measure of host offspring production, survival, successful mating, growth rate (for juveniles) or a measure of host body condition (*e.g.* body mass) or health (*e.g.* anaemia).

RESULTS

Haemocyte counts and parasitism in the field

Pasteuria ramosa-infected *Daphnia* first appeared in early June, and prevalence peaked three times over the season: in early June, late August and late September, achieving a maximum prevalence of 32%, which is likely to be an underestimate as individuals with low level infections can be overlooked. There were also three peaks in the number of circulating haemocytes in the hosts, which occurred at approximately the same times as parasite prevalence peaks (Figure 5.1).

Of the ecological variables tested, only infection status explained a significant amount of the variation in haemocyte counts: healthy wild *Daphnia* had a mean of

1261.52 ± 115.24 haemocytes, whereas their parasite-infected counterparts had 5609.71 ± 814.37 circulating haemocytes, and this pattern was clearly consistent on nearly all sampling dates (Figure 5.1). No other variable, from either the past or future, significantly determined haemocyte counts (Table 5.1). Finally, there was a significant negative correlation between current *P. ramosa* prevalence and *Daphnia* population density two weeks in the future (r_s = -0.51, $p < 0.05$), a pattern that was even stronger four weeks into the future (r_s = -0.74, $p < 0.01$; Figure 5.2).

Early cellular response experiment

Haemocyte counts were obtained from 240 *Daphnia* from 48 replicate jars. I found that *Daphnia* mounted a cellular response to *P. ramosa* exposure: there were 358 ± 37 haemocytes per *Daphnia* in parasite-exposed hosts, and 128 ± 14 haemocytes per *Daphnia* in control (unexposed) hosts ($F_{1,40}$ = 45.43, $p < 0.0001$). The number of circulating haemocytes also depended on the identity of the host genotype ($F_{3,40}$ = 2.93, $p < 0.05$; Figure 5.3), but there was no host genotype-by-parasite exposure interaction ($F_{3,40}$ = 1.46, p = 0.24; Figure 5.3).

Infection experiment – long-term cellular response

Haemocyte counts were obtained from 234 *Daphnia* from 48 jars. *Pasteuria ramosa*-infected *Daphnia* had considerably more haemocytes circulating in their haemolymph: 8458.3 ± 1190.0 for infected and 3164.2 ± 249.3 for healthy hosts (Figure 5.4, $F_{1,63}$ = 29.64, p <0.0001), and haemocyte counts were also affected by host genotype ($F_{3,63}$ = 3.62, $p < 0.05$). These haemocyte counts were consistent with those from wild

Daphnia. There were no significant differences between haemocyte counts from control replicates and uninfected *P. ramosa*-exposed replicates ($F_{1,67} = 0.01$, $p = 0.93$), but the differences between host genotypes remained ($F_{3,67} = 4.40$, $p < 0.01$). Finally, none of the *Daphnia* from control replicates became infected; and, the likelihood of infection in exposed replicates was dependent on the genotype of the host (change in deviance = 26.80, $F_{3,67} = 6.60$, $p < 0.001$).

Ecoimmunology literature search

My Web of Science search yielded 318 research papers, of which 153 were discarded because they were reviews, technical reports or theoretical papers, or did not measure host immune responses. Of the remaining 165, 42% (70/165) only measured host immune activity, 15% (24/165) measured host immune activity and infection status, 27% (45/165) measured host immune activity and host fitness, and 16% (26/165) measured host immune activity, infection status and host fitness.

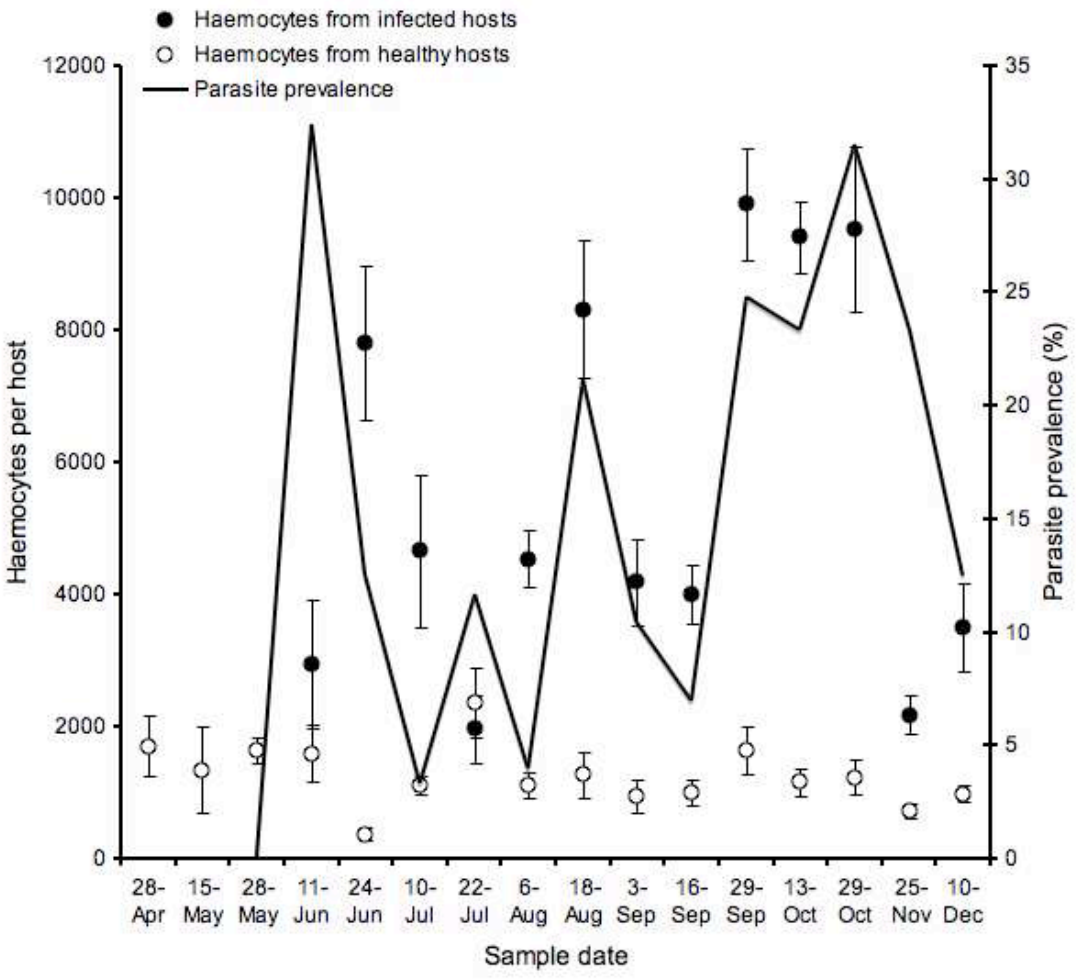

Figure 5.1. Mean number of haemocytes per *Daphnia* (in uninfected and infected groups ± 1 S.E.) and *P. ramosa* prevalence in a natural population in Scotland over a nine month period in 2009.

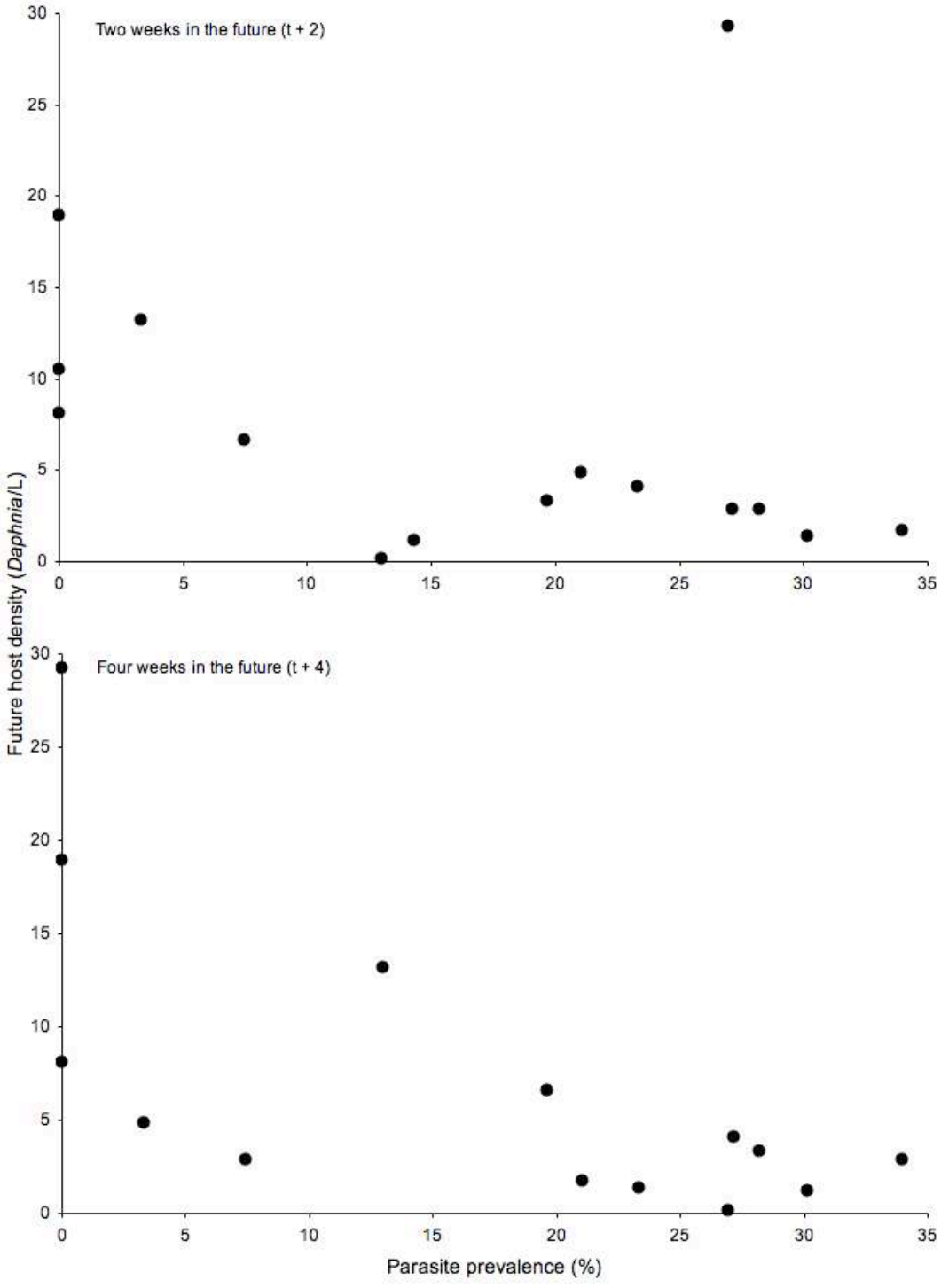

Figure 5.2. Correlations between current *P. ramosa* prevalence and *Daphnia* population density two and four weeks in the future.

Table 5.1. Relationships between (a) current, (b) past and (c) future environmental variation and the number of host circulating haemocytes. *I* is the infection status (infected or healthy), Prev is the population parasite prevalence, Dens is the host population density, Temp is the pond temperature. Sample date was fitted as a random effect in all analyses, but only ever accounted for a small proportion of variance in the data (9.98×10^{-10} for current analysis; 1.47×10^{-8} for past analysis; and 6.52×10^{-10} for future analysis).

Source	Coeff	SE	*t*	*p*
(a) Current				
Intercept	5.401	0.162	33.26	**< 0.0001**
I	-0.605	0.082	-7.25	**< 0.0001**
Prev	0.326	0.398	0.82	NS
Dens	-0.001	0.00011	-0.79	NS
Temp	0.004	0.104	0.48	NS
(b) Past				
Intercept	5.601	0.167	33.35	**< 0.0001**
I	-0.656	0.085	-7.68	**< 0.0001**
Prev	-0.414	0.402	-1.03	NS
Dens	-0.00005	0.0001	-0.47	NS
Temp	0.002	0.01	0.19	NS
(c) Future				
Intercept	5.437	0.102	53.31	**< 0.0001**
I	-0.614	0.084	-7.27	**< 0.0001**
Prev	0.322	0.401	0.80	NS
Dens	0.00002	0.00009	0.17	NS

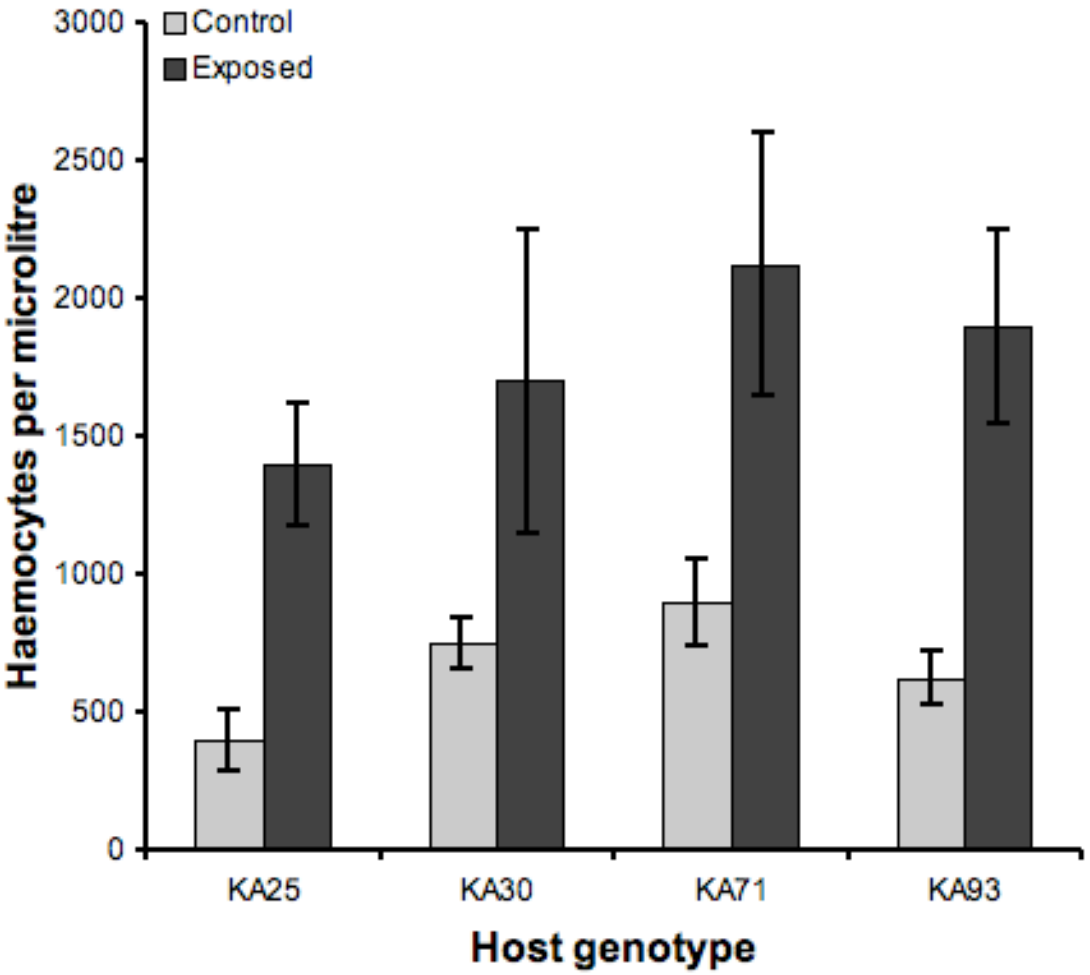

Figure 5.3. Mean number of haemocytes from parasite-exposed and control *Daphnia* 5 hours after treatment exposure. Counts are expressed per *Daphnia* ± 1 S.E. There were six replicates per treatment, per genotype.

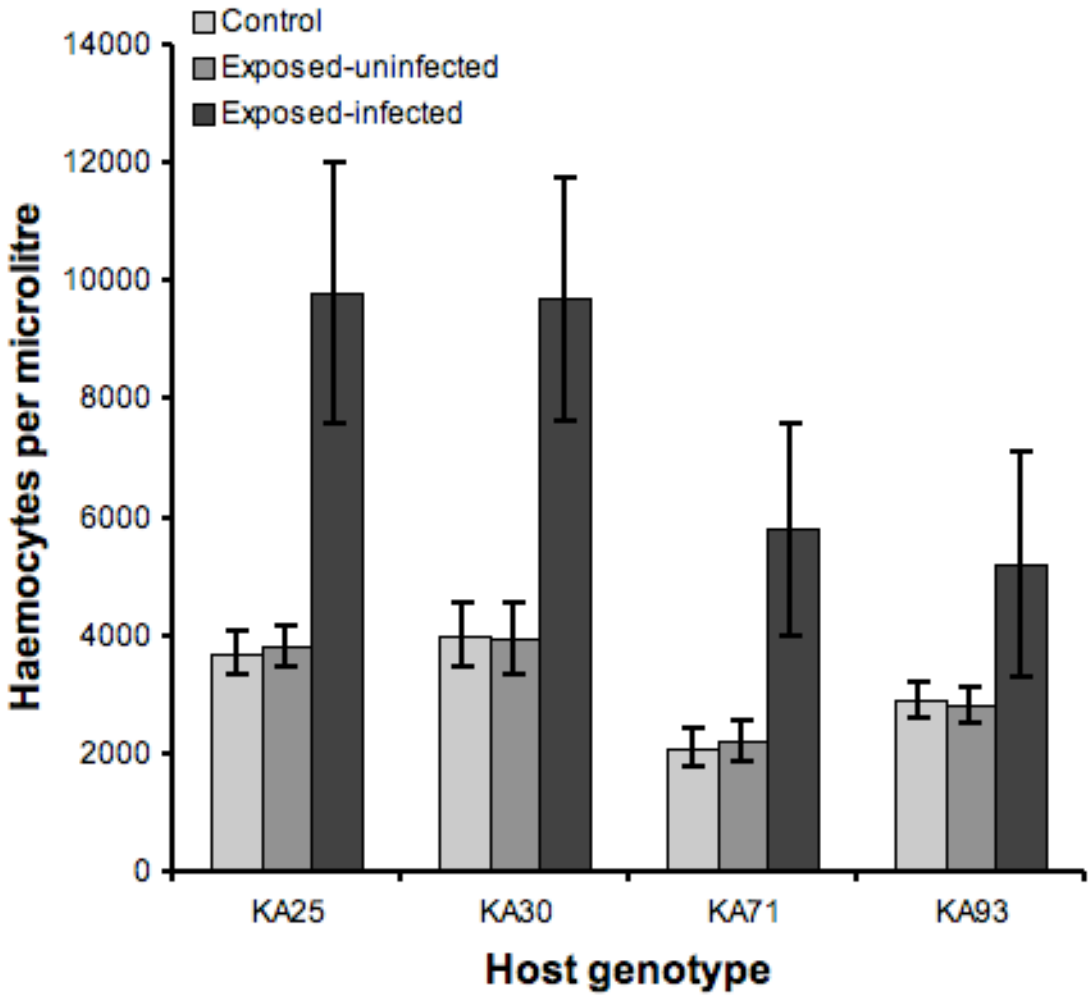

Figure 5.4. Mean number of haemocytes per *Daphnia* from parasite-unexposed (control), parasite-exposed but uninfected and parasite-exposed and infected *Daphnia* 21 days after treatment exposure. Counts are expressed per *Daphnia* ± 1 S.E. There were six control replicates and 12 parasite-exposed replicates per genotype.

DISCUSSION

The number of circulating haemocytes in wild *Daphnia magna* was far higher in infected than in uninfected hosts. Thus, this putative immune trait is a reporter for infection with the sterilizing bacterium, *Pasteuria ramosa* (Figure 5.1). Further, since infection results in host sterilisation, elevated haemocyte number reflects a termination in the host's fitness potential. Greater immune activity is sometimes associated with strong defence capability: either a high potential to resist parasites, or as evidence of a successful defence in the past. However, as observed here, immune activity can also be indicative of high current parasite burden, and hence a low fitness potential (AULD *et al.* 2010; LINDSEY and ALTIZER 2009; VINEY *et al.* 2005). Drawing this conclusion required simultaneously diagnosing infection and its fitness consequences whilst measuring the immune response (GRAHAM *et al.* 2011). With comprehensive measurement, it is possible to draw conclusions about what high immune activity means for host fitness, and in the present case a large haemocyte response can be equated with being (genetically) dead because *P. ramosa* sterilises its hosts. Indeed, the consequences of this sterilisation were evident at the population level, as high parasite prevalence predicted lower host population density in the future (Figure 5.2), consistent with previous studies (DECAESTECKER *et al.* 2005; DUNCAN *et al.* 2006; LITTLE and EBERT 1999).

One of the aims of this study was to determine if the cellular response patterns observed in the laboratory were mirrored in noisy natural environments, where environmental conditions, and thus host condition, should be very different from those in the laboratory. Little is known about just how context-dependent individual immune

responses might be (exceptions include (HARVELL *et al.* 2001; KLEMOLA *et al.* 2007; SEPPAELAE and JOKELA 2010). For example, to what degree do the intricate mechanisms of immunity, which are typically studied under tightly controlled conditions and in a limited range of genetic backgrounds, manifest under more stressful conditions or in the wild? In this study, what was true for *Daphnia* in the field was also true for those that had been experimentally exposed to *P. ramosa* and reared under controlled laboratory conditions. Moreover, although *Daphnia* haemocyte counts fluctuated over the course of the field season (Figure 5.1) they remained a robust marker for parasitism; variation in parasite prevalence host density or pond temperature did not reduce the strength of the signal, even when lags were included in the analyses.

Still, variation in environmental conditions is known to substantially affect infection outcome in many host-parasite systems (see LAZZARO and LITTLE 2009; VALE *et al.* 2008). For example, based on past research on temperature (MITCHELL *et al.* 2005; VALE and LITTLE 2009; VALE *et al.* 2008), I expected, but did not observe, a relationship between temperature and infection levels (that is, beyond the superficial observation that epidemics tend to occur in the summer months: (DUNCAN *et al.* 2006). Additionally, because elevated temperature also favours an increased rate of development and reproduction in healthy *Daphnia,* I also expected, but did not observe, some linkage between temperature and population growth. An extensive study in the *Daphnia dentifera-Metschnikowia bicuspidata* system has also failed to detect an effect of temperature on disease phenomena in the wild (DUFFY *et al.* 2009). In both instances, effects may be hard to detect because of joint increases in host and parasite metabolism with increasing temperature.

Genetic differences for the cellular response to *P. ramosa* were evident in the experimental studies presented here, but each of the host clones showed a significant cellular response (and some infection), and difference between clones was one of magnitude. In a previous study on a different population (AULD *et al.* 2010), some host genotypes showed no response at all, and these were the ones that also showed complete resistance (there were no successful infections in these genotypes). This contrast between populations confirms that a strong immune response is associated with, though does not cause, susceptibility. In particular, the hosts used in the previous study show very dichotomous patterns of variation for susceptibility (and hence all-or nothing cellular responses, at least to the single parasite strain used in that study). In the current population, although there is only subtle variation in how readily different host clones succumb to infection, all hosts are ultimately susceptible to the mixed parasite spore solution used (and thus all were expected to show a cellular response, as observed).

The results are thus compatible with a previously proposed heuristic model of defence in *Daphnia:* for successful infection to occur, *P. ramosa* spores need to (1) pass from the *Daphnia*'s gut into its haemocoel, and then (2) avoid haemolymph-based host immune effectors (AULD *et al.* 2010). Strong resistance in *Daphnia* appears to be based upon parasites *not* passing the gut wall, and this could be based on (so far measured) specific recognition factors that either do not allow penetration, or suppress the parasite at a very early stage. In susceptible hosts, which lack the specific recognition factors that lead to resistance, parasites gain entry and haemocyte numbers rapidly increase in response. Thus, an increase in haemocyte numbers is not causally linked to susceptibility/resistance- haemocyte numbers report infection (although infection only occurs in susceptible genotypes, and so haemocyte numbers are indirectly associated

with susceptibility). One might reason that haemocyte activity may still be beneficial when other defences fail: perhaps by mobilising haemocytes, susceptible hosts delay their sterilisation? However, findings from my earlier work reject this hypothesis: in infected hosts, haemocyte number was always negatively associated with host fitness (Chapter 3). Nevertheless, haemocytes may vary in their efficacy between genotypes, and functional readouts of haemocyte activity (*e.g.* Nitric Oxide production) could greatly refine the work on the *Daphnia* immune response.

In general, studies linking immune activity with infection status, and host and parasite fitness, are essential for our understanding of host-parasite coevolution (GRAHAM *et al.* 2011), but I acknowledge that is not always possible for a study to be comprehensive. It is simply important to bear in mind that studies using a restricted suite of measurements should cautiously avoid making assumptions about the functional significance of immune activity. Specifically, studies that measure immune activity and infection (but not host fitness) will often overlook the possible role of immunopathology; studies that measure immune function and host fitness (but do not assess parasite burden) may overlook the possible role of infection in causing variation in host immune capabilities.

CHAPTER 6

Parasite evolution associated with decreasing host availability in the *Daphnia-Pasteuria* system

I studied how parasite infection traits change over the course of a growing season in a natural population of the crustacean *Daphnia magna* and its sterilizing bacterial parasite *Pasteuria ramosa*. The number of parasite transmission spores per infected host increased ten-fold over the course of the growing season, and this increase was accompanied by a decline in host density. This change in parasite intensity could be attributable either to genetic changes, *i.e.* evolution, in the parasite population such that faster growing strains were selected late in the season, or to environmental or demographic changes that fostered parasite growth independent of any genetic changes. To test this, I preserved field-collected parasite spores throughout the season, and later exposed a set of hosts to a fixed dose of these spores under controlled laboratory conditions. Parasites collected late in the season were more infective and grew more rapidly than parasites collected early in the season. Thus, I was able to demonstrate that changes in wild parasite infection traits were due to adaptive evolution, and not due to non-genetic effects.

INTRODUCTION

Parasites and pathogens offer excellent examples of evolution in action. Their short generation times mean they can be observed evolving in the laboratory (MORGAN *et al.* 2005; RAINEY 2004), and in the real world, as evidenced by the evolution of drug resistance (MARCHESE *et al.* 2000) and vaccine escape mutants (BANGHAM *et al.* 1999). Emerging diseases and epidemics also often stem from evolutionary events (MORSE 1994; SMITH *et al.* 2009). Parasite evolution has been given substantial theoretical treatment by a set of models generally referred to as evolution of virulence models. Many such models have focussed on identifying conditions that can favour more virulent parasites (in particular parasites that grow faster and cause more harm), and have, for example, suggested that natural selection can favour increased parasite growth (and hence virulence) when the density of susceptible hosts is high (BULL 1994; DAY and GANDON 2007; EWALD 1994), but see (ALIZON *et al.* 2009). This is because growing fast and killing your host rapidly is a reasonable strategy when there are other hosts immediately available to move on to.

For example, in a fig wasp-nematode system, increased opportunities for parasite transmission is associated with a higher frequency of hosts suffering infection with multiple nematode strains (HERRE 1995). The accompanying increase in within-host competition between these nematode strains leads to higher average parasite growth as each strain is racing to sequester more of the limited resource; higher virulence accompanies this increased within-host growth rate (HERRE 1995; see also BEN-AMI *et al.* 2008a; MASSEY *et al.* 2004). Another study has demonstrated that an abundance of hosts selects for fast-infecting parasites, whereas host rarity selects for

slower infecting and more fecund parasites, but infection rate trades off against other fitness-related traits, namely, within-host growth and survival (CROSSAN *et al.* 2007). Nevertheless, whilst there is limited field-collected and empirical data supporting the idea that host density selects on parasite traits, studies combining these two approaches are lacking.

Here, I used the crustacean *Daphnia magna* and its sterilizing bacterial parasite *Pasteuria ramosa* to examine parasite evolution in the field. First, I studied changes in parasite prevalence (the proportion of infected hosts within a population), infection intensities (the number of parasites growing within each infected host) and the number of haemocytes circulating in the host across a growing season in the wild. These parameters were associated with host availability (*i.e.* host population densities) and other ecological variables. To disentangle whether any observed changes in the field were due to parasite evolution or non-genetic effects, I exposed, under common garden conditions in the laboratory, a set of standard host genotypes to parasites collected (and then stored frozen at -20ºC) from different times during the field season. Thus I combined observations of parasite change in the field with experimental verification of the cause of that change in the laboratory.

MATERIALS AND METHODS

The Daphnia magna-Pasteuria ramosa *model system*

Daphnia magna is a cyclically parthenogenetic freshwater planktonic crustacean that inhabits shallow freshwater ponds. Like most organisms, *Daphnia* are host to a number of parasites (EBERT 2005; EBERT 2008; GREEN 1974), including the obligate

microparasite, *Pasteuria ramosa*. *Pasteuria ramosa* is a spore-forming bacterium that is transmitted horizontally from the corpses of previously infected hosts (EBERT *et al.* 1996). Infection occurs when *Daphnia* filter-feed; they take in the transmission spores along with their food, and once in the host, the *P. ramosa* spores go through a 10-20 day developmental process, resulting in many millions of transmission spores that are released on the death of the *Daphnia*. The process of parasite development and reproduction uses up resources that would otherwise be used for host reproduction, and *Daphnia* are almost always sterilized as a direct result of infection with *Pasteuria ramosa*. *Pasteuria*-infected *Daphnia* can be easily identified by eye: they have obvious red bacterial growth in their haemolymph; they are usually larger and lack developed ovaries and eggs in their brood chamber.

The *Daphnia-Pasteuria* model has been used in many studies of parasite fitness because infection status is easily determined and transmission stages (henceforth transmission spores) are only released on the death of the host (EBERT *et al.* 1996). Good estimates of both parasite lifetime reproductive success and transmission potential can therefore be obtained by counting the number of transmission spores from infected hosts (see EBERT *et al.* 2000; JENSEN *et al.* 2006; LITTLE *et al.* 2008; VALE and LITTLE 2009). Also, the number of circulating haemocytes per *Daphnia* rapidly increases soon after exposure to infectious *P. ramosa* spores (*i.e.* there is a cellular response *sensu* METCHNIKOFF 1884, but see AULD *et al.* 2010), and baseline haemocyte number is greater in *P. ramosa* –infected as opposed to healthy hosts (AULD *et al.* submitted; Chapter 5). It remains to be determined whether haemocyte number is associated with the number of *P. ramosa* transmission spores.

Field haemocyte and parasite spore counts

Daphnia magna were sampled from three fixed points around a pond at Kaimes Farm, Leitholm, Scottish Borders (2˚20.43'W, 55˚42.15'N) twice per month between April and November 2010. This pond is approximately 500 m from the pond surveyed in my previous field study (Chapter 5; AULD *et al.* submitted). Adult *Daphnia* from each sample point were collected by sweeping a net with an opening of 0.063m^2 through one metre of pond water, and were then grouped according to infection status. Hosts from each grouping were placed five at a time in a cell extraction chamber with 4.0 μl of ice-cold anticoagulant buffer (98 mM NaOH, 186 mM NaCl, 17 mM EDTA and 41 mM citric acid, pH adjusted to 4.5: LAVINE *et al.* 2005) and their hearts were pierced using a 25-gauge needle (BD Microlance, Drogheda, Ireland), causing haemolymph to pool into the buffer. This haemolymph-buffer solution was then transferred into 0.5 ml Eppendorf tubes and placed on ice for the hour-long journey back to the laboratory. Cadavers were also transferred into 0.5 ml Eppendorf tubes.

In the laboratory, each of the haemolymph samples was mixed thoroughly using a pipette, and 2 μl were placed in a fertility counting chamber (0.001 mm^2 × 0.100 mm depth, Hawksley, Lancing, Sussex, UK), and the number of haemocytes was counted. These counts were converted to number of cells per microlitre of haemolymph-buffer solution. The cadavers were homogenized in 500 μl of ddH_2O, and 8 μl were placed in a Neubauer (Improved) counting chamber (0.0025 mm^2 × 0.1 mm depth), and the number of *P. ramosa* transmission spores (an estimate of parasite fitness) was determined. These spore solutions were then frozen at -20ºC.

At each sampling location, water temperature was measured using a digital field thermometer (HANNA instruments HI93510). The population density of *Daphnia* was

estimated from live collections back in the laboratory: the number of infected adults, healthy adults and juveniles were counted and the density (in *Daphnia*/litre) of each life stage was this count divided by the volume of water the net was passed through (water volume = 0.063 m^2 (net area) × 1m (the sweep distance)). *Pasteuria ramosa* infection was assessed in the adult portion of all subsamples. Infection was usually assessed by eye, but in the occasional ambiguous case, individuals were crushed under a glass coverslip on a microscope slide, and then examined under a transmission microscope for the presence of *P. ramosa* spores.

Experimental setup

The experiment was designed to test whether: (1) the ability of *P. ramosa* to infect (2) its reproductive success in infected hosts and (3) its ability to elicit a host cellular response changed over the course of the epidemic. Methods were similar to Auld *et al.* (2010). A test set of four standard *Daphnia* genotypes (named KA40, KA53, KA62 and KA81)(*sensu* BURDON and JAROSZ 1991; BURDON and ROBERTS 1995) were maintained as independent replicates. These host genotypes were chosen because they suffer varying levels of infection when exposed to *P. ramosa* spores from my study population. They were founded from laboratory-hatched ephippia collected from a local pond (ALLEN and LITTLE in press) and kept in the laboratory in a state of clonal reproduction for three generations, to minimize variation in condition. Hosts were kept in groups of five in jars containing 200 ml of artificial medium (KLUTTGEN *et al.* 1994) and fed 5.0 ABS of chemostat-grown *Chlorella vulgaris* algal cells per day (ABS is the optical absorbance of 650 nm white light by the *C. vulgaris* culture). Their medium was

changed three times per week, and after the *Daphnia* had offspring, and the jars were incubated at 20°C on a 12L:12D light cycle. Second clutch neonates formed the experimental replicates in each of the two experiments.

The parasite spores used here were from samples collected during the field study on June 8th, July 6th, August 3rd, August 17th, September 2nd and September 16th 2010. These are the same spores used for the spore counts shown in Figure 6.3, and were frozen (at -20ºC) within three hours of collection. On the day of experimentation, I defrosted the spore samples and for each sample date, I made a single spore solution consisting of equal numbers of spores from replicate samples. Thus, there were six spore solutions in total. These were then diluted with ddH_2O until each final solution was at a concentration of 1,000,000 spores per ml.

Replicates were divided between seven treatments: controls, or exposure to one of the six parasite samples. There were 12 replicates per parasite treatment, per genotype. Experimental replicates were kept in the same conditions as maternal generations until at least three of the five *Daphnia* deposited eggs in their brood pouch, at which point they were ready for parasite exposure. Parasite treatments were carried out as follows: for each replicate, the five adult *Daphnia* were placed in the well of a 24-well plate (Costar, Corning Inc., NY, USA). Parasite spore solutions were thawed, thoroughly mixed with a pipette and the number of spores was determined using a Neubauer (Improved) counting chamber (0.0025 mm^2 × 0.1 mm depth). Replicates assigned to the parasite-exposed treatments then received 50 000 *P. ramosa* spores (50 μl), and control replicates received an identical volume of homogenised healthy *Daphnia* as a placebo.

Treatment exposure lasted for five hours, after which the *Daphnia* were removed from the cell plate and washed in artificial medium. Four of the five hosts in each replicate were dried on a paper towel and then placed on a glass Petri dish. Their hearts' were then pierced with a 25 gauge needle (BD Microlance, Drogheda, Ireland), and from each of the four *Daphnia,* 1.0 μl of haemolymph was pipetted and mixed with 4 μl of anticoagulant buffer. Haemocytes were then counted using methodology described earlier. It is important to note that this measure of host cellular immune activity in the laboratory is different to the number of haemocytes recorded in the field: the initial haemocyte number in the experiment reflects a hosts response to the initial stages of infection, whereas the number of haemocytes documented in the field reflects host cellular immune activity once infection is (or is not) established. In any case, both measures yield similar information regarding infection in the *Daphnia-Pasteuria* system: an increase in haemocyte number following parasite exposure predicts likely future infection (AULD *et al.* 2010); Chapter 2; Chapter 3; Chapter 4; Chapter 5), and the number of haemocytes is significantly higher in already infected hosts (Chapter 5).

The fifth *Daphnia* from each replicate was placed singly in a small jar with 60 ml of artificial medium. Medium was refreshed three times per week, and after the *Daphnia* had a clutch of offspring. The number of offspring clutches was recorded for each *Daphnia,* as well as infection status (infected or not). The experiment was terminated on day 30, when all surviving hosts were placed in a 1.5 ml Eppendorf. Counts of *P. ramosa* transmission spores in each infected host were determined as follows: individual *Daphnia* were homogenized with 100 μl of ddH_2O, and two independent counts were made from the resulting suspension in a Neubauer (Improved) counting chamber (0.0025 mm^2 × 0.1 mm depth) under 40 × magnification.

Analysis of field data

All data were analysed using general linear models implemented in the R statistical package (IHAKA and GENTLEMAN 1996; R 2005), and for all models, the significance of the predictor variables was examined hierarchically using a stepwise backward model reduction procedure (CRAWLEY 2007).

First, I analysed the parasite spore count data. This was done by testing the effects of temperature, parasite prevalence, $\log_{10}$[host density] and $\log_{10}$[number of haemocytes] on the $\log_{10}$-transformed parasite spore counts from infected hosts; all two-way interactions were also included as explanatory variables. I could not include sample date in this analysis, because it was so closely associated with $\log_{10}$[host density]. Therefore, as a secondary analysis on data from infected hosts only, I tested whether $\log_{10}$[parasite spores] varied with sample date by fitting a model with just sample date as a fixed factor. Sample date was fitted as a factor because the parasite spore counts came from *Daphnia* that were removed from the pond, meaning the data fell into discrete populations.

Next, I analysed the number of haemocytes circulating in the host's haemolymph, my measure of host immune activity. I did this by testing the effects of host infection status (infected or not), pond temperature, parasite prevalence and $\log_{10}$[host density] on $\log_{10}$-transformed haemocyte count data; all two-way interactions again also included as explanatory variables.

Analysis of experimental data

First, I analysed parasite infection traits. Using data from only parasite-exposed hosts, I analysed the probability of infection by fitting a generalized linear model with a binomial error structure and a logit link function to the infection data, with host genotype, parasite sample and their interaction as explanatory variables. Then, using data from infected hosts only, I analysed the number of parasite transmission spores using a two-way ANOVA, including the same explanatory variables as before.

Next, I examined whether exposure to the field-collected *P. ramosa* samples elicited a cellular response in the hosts. To do this, I performed a two-way ANOVA, where host genotype, parasite exposure (exposed or non-exposed control) and their interaction were fitted as fixed factors. Then, using data from parasite-exposed hosts only, I examined whether parasite sample had an effect on haemocyte counts, again using a two-way ANOVA, but with host genotype, parasite sample and their interaction fitted as fixed factors. Finally, I examined whether host genotype or parasite sample (including controls) had an effect on early host mortality (rate of death before day 30). To do this, I performed a one-way ANOVA with both host genotype and parasite sample (including controls) as fixed factors. In all cases where the data were non-orthogonal, type III sums of squares were used.

RESULTS

Field data

Pasteuria-infected *Daphnia* were observed from early June until mid-September, and parasite prevalence (the proportion of hosts that became infected) peaked twice: in early

July and in mid August (Figure 6.1). There was a negative association between the number of parasite transmission spores per infected host and total host density (Table 6.1; Figure 6.2), and the number of parasite transmission spores negatively correlated with host haemocyte number, but only when parasite prevalence was low, *i.e.* there was a significant interaction between parasite prevalence and haemocyte number (Table 6.1). The number of parasite transmission spores per infected host also increased dramatically over the season ($F_{7,13} = 8.04$, $p < 0.001$): infected hosts collected in late September had over ten-fold more spores than those collected in early June (Figure 6.3). Finally, confirming previous work (Chapter 5) parasitized *Daphnia* had consistently more circulating haemocytes than their healthy counterparts ($F_{1, 7} = 153.01$, $p < 0.0001$), but haemocyte number was not associated with pond temperature, parasite prevalence or host density.

Experimental data

Analyses of parasite-exposed hosts demonstrated that the probability of a host suffering infection depended on the parasite sample date (Table 6.2; Figure 6.4). Analyses of spore count data from infected hosts only indicated that the final number of *P. ramosa* transmission spores depended on the host genotype and the parasite sample date (Table 6.2; Figure 6.5). *Daphnia* mounted a cellular response to *P. ramosa*: after treatment exposure, parasite-exposed hosts had 1282 ± 53 haemocytes, whereas unexposed controls had 604 ± 43 haemocytes (see Table 6.3). The strength of this cellular response to parasite exposure also depended on the specific combination of host genotype and parasite sample date (Table 6.3; Figure 6.6). I also observed a small

amount of mortality in this experiment (< 4 % of hosts died before day 30), but this mortality did not depend on the identity of the host genotype or the parasite treatment (Table 6.2).

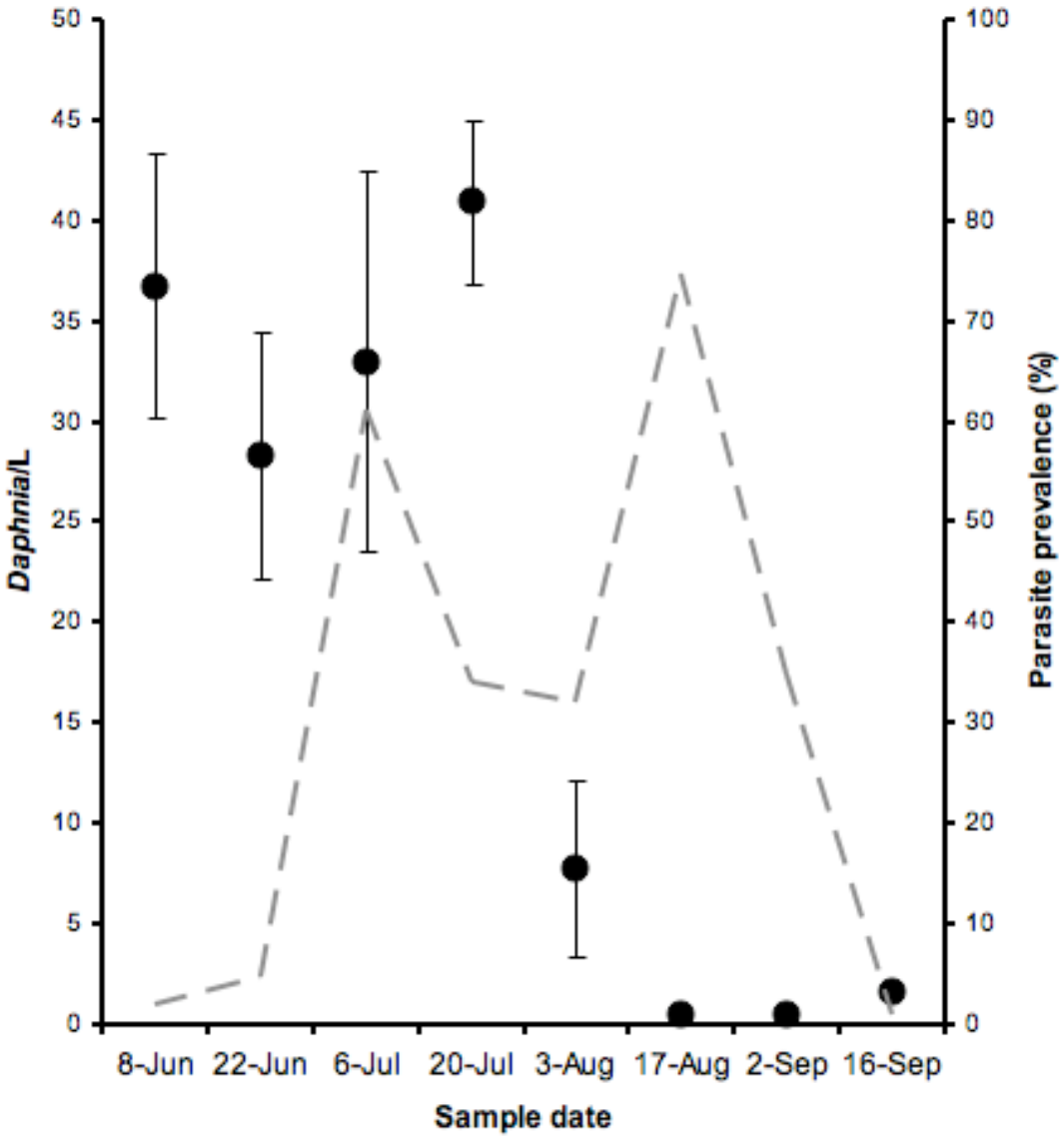

Figure 6.1. Mean *Daphnia* population density (±1 S.E., bold circles) and mean *P. ramosa* prevalence (grey dashed line) over time in a natural population in Scotland.

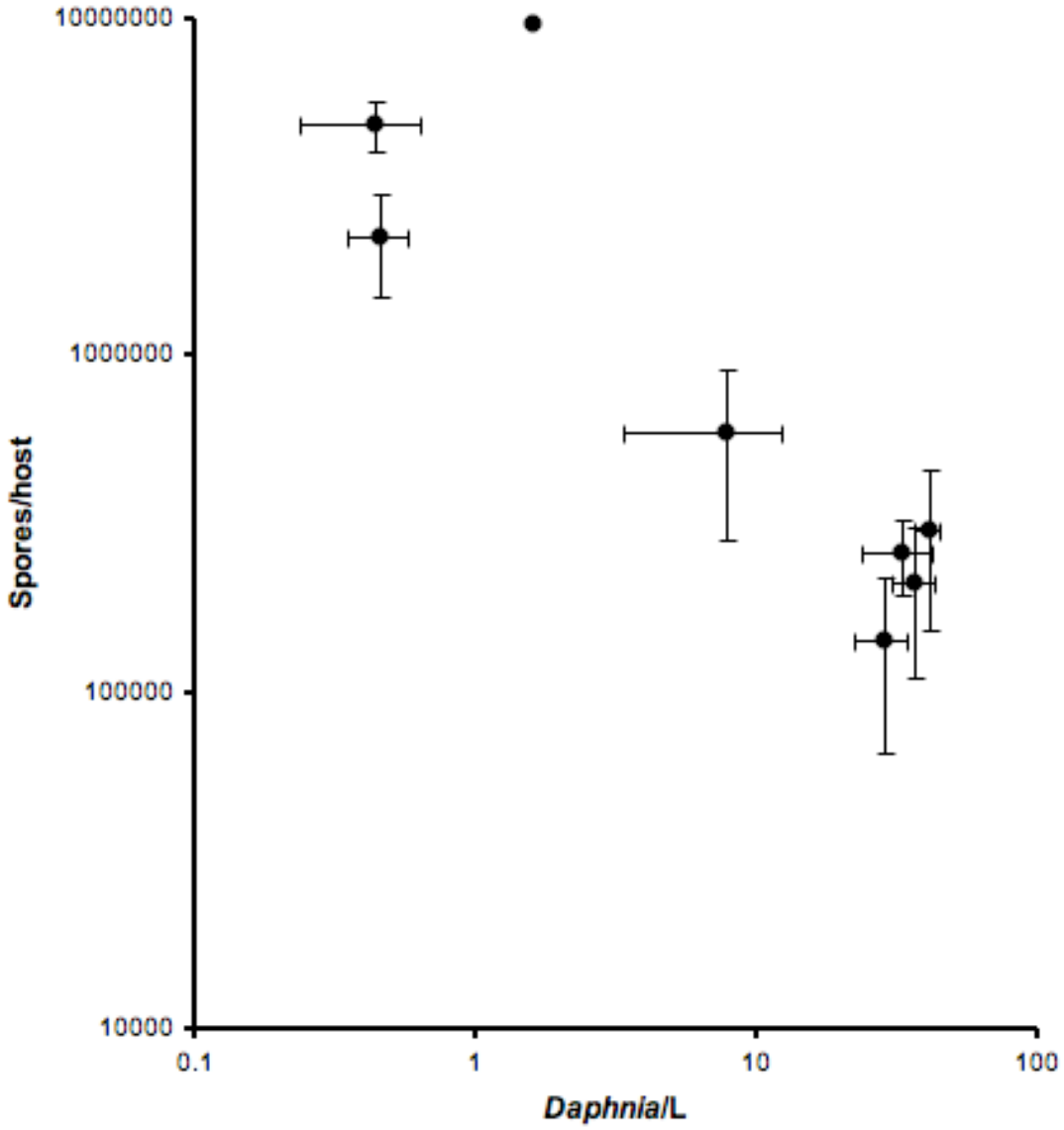

Figure 6.2. Association between the numbers of *P. ramosa* transmission spores per infected host and host population density (both ±1 S.E.) in a natural population in Scotland.

Table 6.1. Analysis of how parasite transmission spore number is associated with population-level parasite prevalence, host density and host haemocyte number.

	Coef	SE	DF	*t*	*p*
Log_{10}[transmission spores]					
Intercept	-3.68	2.45	9	-1.5	0.17
P. ramosa prevalence	21.51	5.22	9	4.12	**< 0.01**
Log_{10}[*Daphnia* density]	-0.53	0.09	9	-5.65	**< 0.001**
Log_{10}[haemocyte number]	2.84	0.68	9	4.15	**< 0.01**
Prevalence x Log_{10}[haemocyte number]	-5.83	1.41	9	-4.13	**< 0.01**

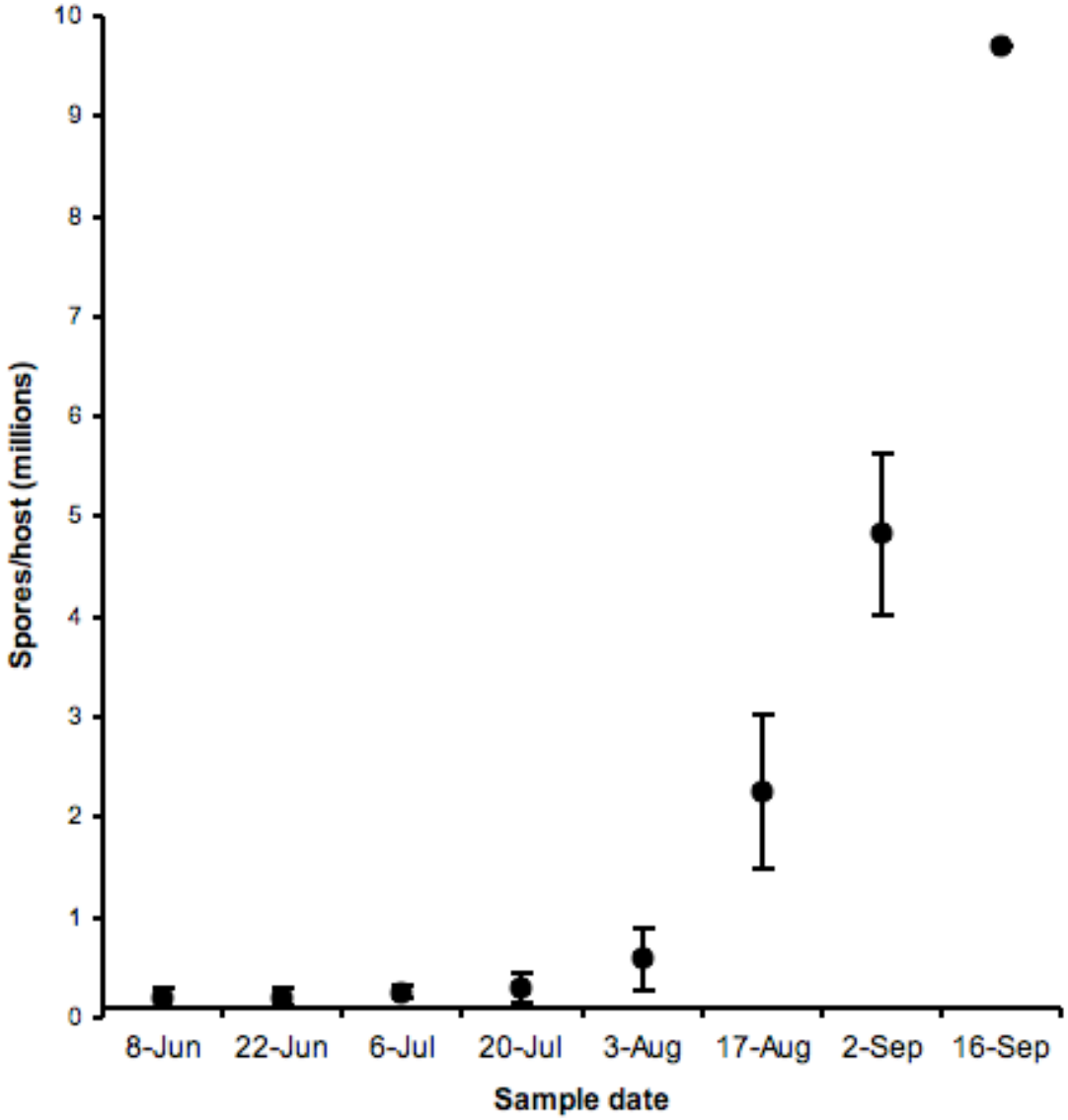

Figure 6.3. Changes in the number of *P. ramosa* transmission spores per infected host (±1 S.E.) over time.

Table 6.2. Analysis of the effects of host genotype and parasite sample date on the probability of infection in parasite-exposed hosts and the number of parasite transmission spores in infected hosts in four genotypes of *Daphnia.*

	DF	LR-χ^2	*p*
Probability of infection (parasite-exposed hosts)			
Host genotype	3	0.70	0.87
Parasite sample	6	133.829	**< 0.0001**
Host genotype x Parasite sample	18	16.752	0.54
Error	278		
	DF	*F*	*p*
Log_{10}[transmission spores] (infected hosts)			
Host genotype	3	14.28	**< 0.0001**
Parasite sample	4	7.20	**< 0.0001**
Host genotype x Parasite sample	10	1.01	0.44
Error	85		
	DF	*F*	*p*
Host mortality (before day 30)			
Host genotype	3	0.26	0.85
Parasite sample	5	0.39	0.83
Error	2		

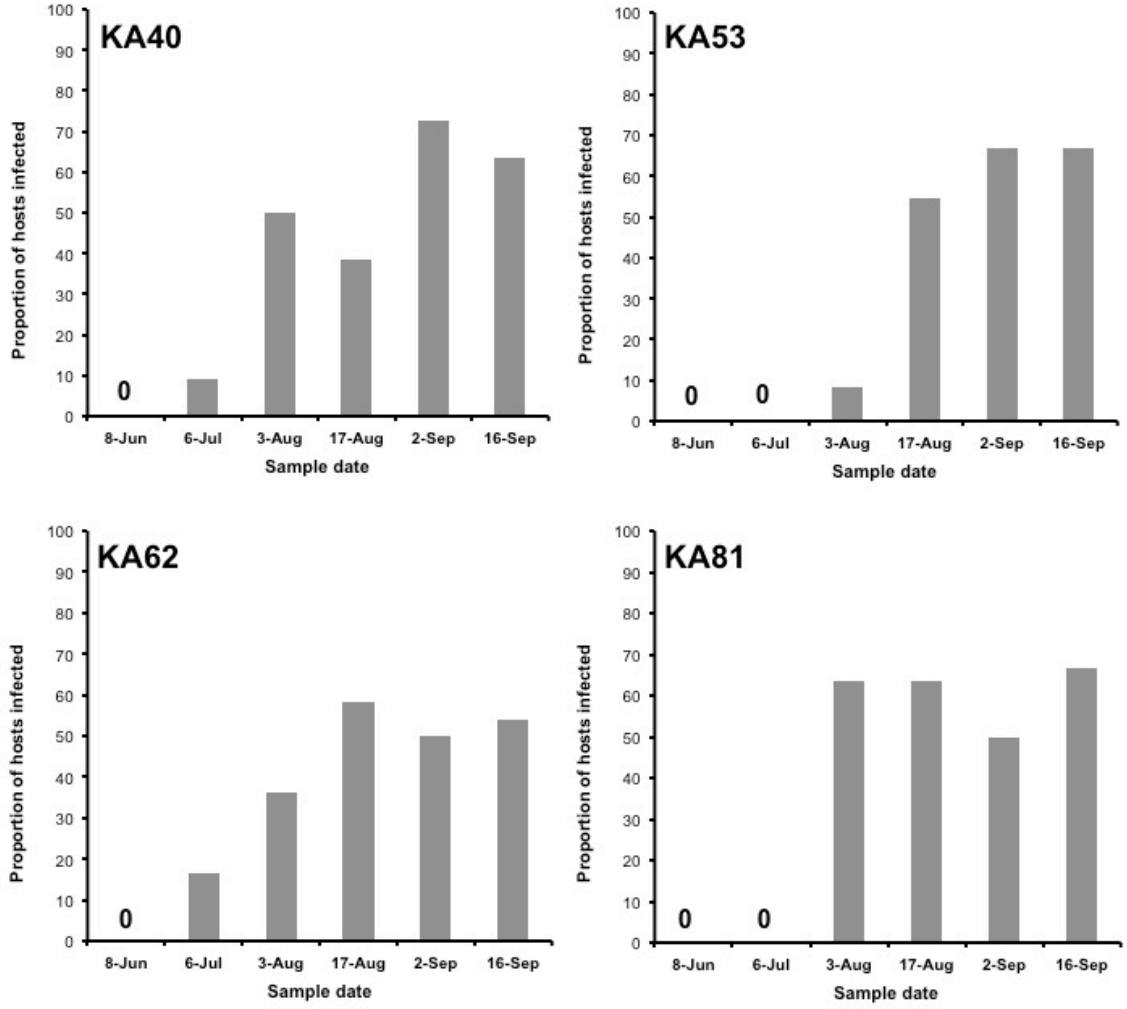

Figure 6.4. Proportion of infected Daphnia in four host genotypes exposed to parasite samples from a wild population. Zeroes indicate treatments where no infections occurred.

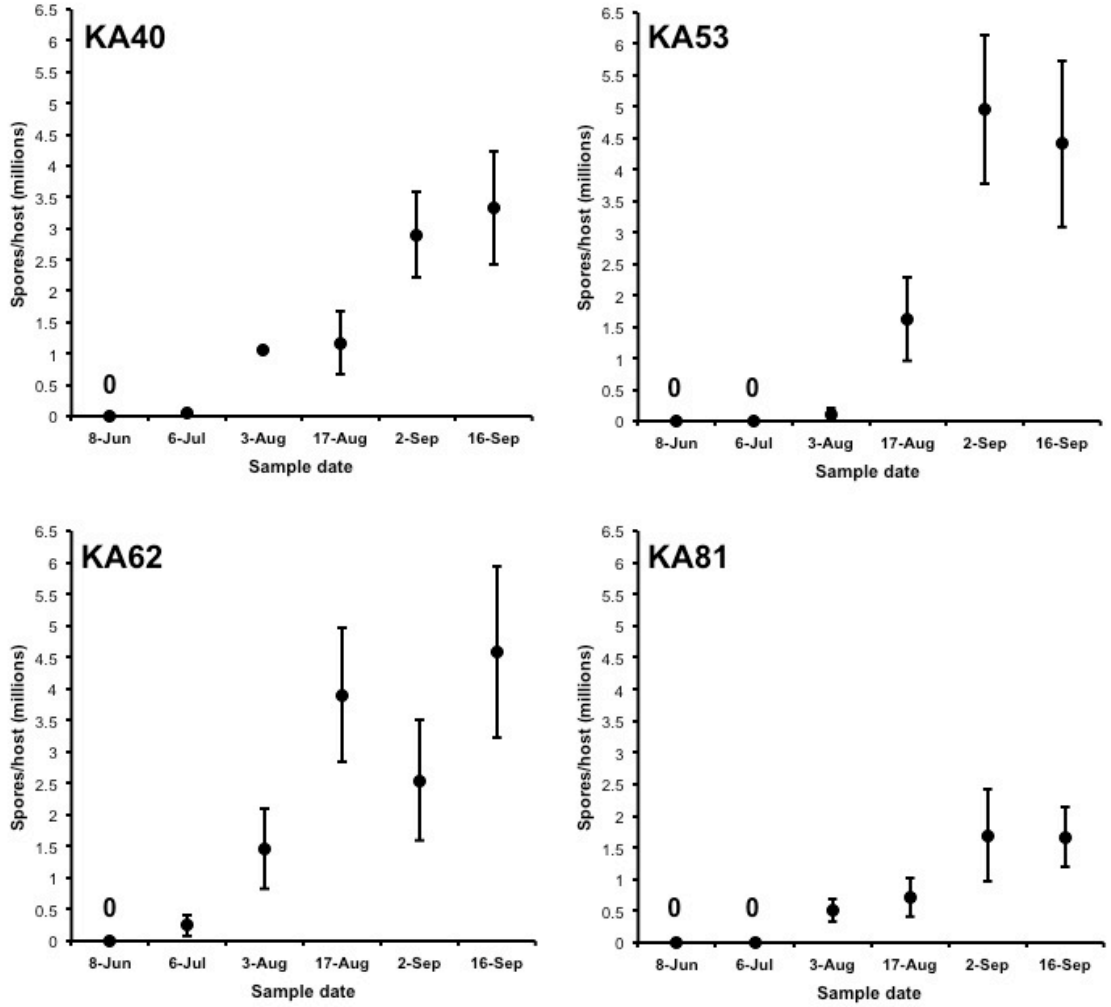

Figure 6.5. Number of *P. ramosa* transmission spores per infected host (± 1 S.E.) in four host genotypes exposed to parasite samples from a wild population. Zeroes indicate treatments where no infections occurred.

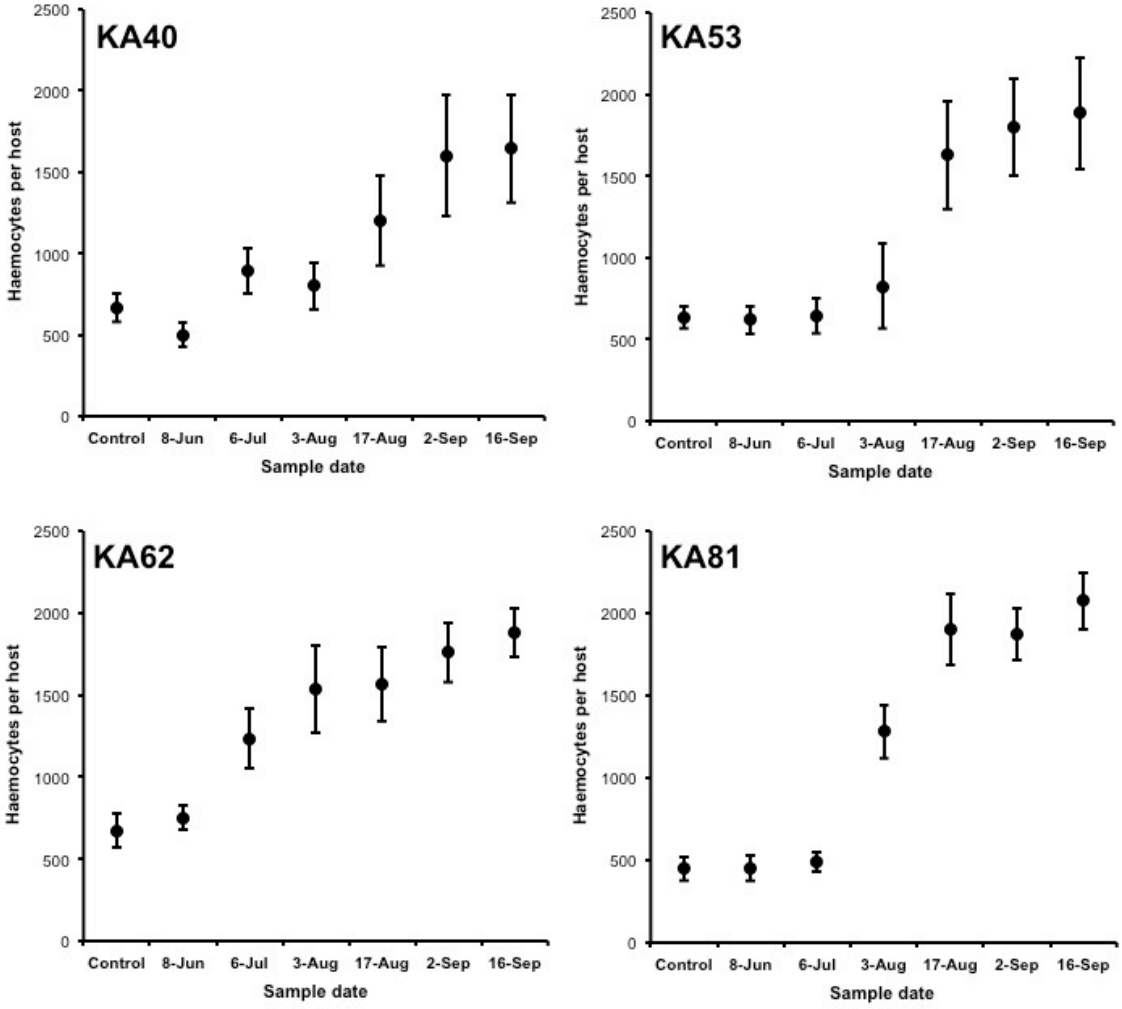

Figure 6.6. Number of haemocytes per host (± 1 S.E.) in four host genotypes exposed to either a placebo (Control) or parasite samples from a wild population.

Table 6.3. Analysis of the effects of controlled exposure to field-sampled *P. ramosa* and the date when that sample was taken on the number of circulating haemocytes in four genotypes of *Daphnia.*

	DF	*F*	*p*
Log10[haemocytes] (all hosts)			
Host genotype	3	3.5719	**< 0.05**
Parasite exposure	1	27.015	**< 0.0001**
Host genotype x Parasite exposure	3	2.1058	0.10
Error	328		
	DF	*F*	*p*
Log10[haemocytes] (parasite-exposed hosts)			
Host genotype	3	6.1603	**< 0.001**
Parasite sample	5	22.7994	**< 0.0001**
Host genotype x Parasite sample	15	2.0322	**< 0.05**
Error	263		

DISCUSSION

From my survey of parasite infection traits in a wild population, I found the number of parasite spores within an infected host was 100 times greater in the autumn than in late spring (Figure 6.3), and accompanying this, host density declined from 30 to 40 hosts per litre to 1.0 or fewer (Figure 6.2). In addition, I observed substantial fluctuation in parasite prevalence, including two peaks where 60-70% of adult host were infected (Figure 6.1), and that haemocyte counts were significantly higher in parasitized *Daphnia* than in their healthy counterparts. Whilst these findings from the field study are consistent with evolution in the parasite population, it could also be that other unrecorded non-genetic factors were influencing the numbers of parasite transmission spores in infected hosts. For example, spore counts from the end of the epidemic may be higher because they are from older hosts that have been infected for longer (allowing for more within-host parasite growth).

Therefore, I brought field-collected parasites (from which the transmission spore counts were made) into the laboratory throughout the summer and preserved them at -20ºC until I could perform an experiment addressing whether parasites from different time points expressed different trait values in a common garden. Under the hypothesis that the parasite population evolved, I predicted that experimental hosts that became infected with parasites from the end of the season would have the highest spore counts. I also predicted that infectivity would be highest in parasite samples preceding the dates when parasite prevalence peaked in the field (the 6th of July and the 17th of August). The predicted lag between peak spore infectivity and the peak in field parasite prevalence is based on the time it takes for *P. ramosa* infection to fully develop.

Finally, I expected the parasite to be most immunostimulatory (elicit the strongest host cellular response on exposure) at times of peak spore infectivity, as my previous study suggests the magnitude of host cellular response reflects the number of *P. ramosa* spores to pass from the host's gut to its haemolymph (Chapter 3).

We found that parasite infectivity, capacity for immunostimulation and within-host growth of transmission spores depended on the date when the parasite spores were initially sampled from the wild (Table 6.2). These findings confirm that phenotypic change in the parasite is likely due to evolution over the course of the epidemic. However, they did not meet my predictions entirely, as all three parasite traits increased in magnitude across the season. Notably, peak infectivity in the laboratory did *not* coincide with peak parasite prevalence in the field (Figure 6.4, Figure 6.1). Further, the strength of host cellular response to a particular parasite sample mirrored parasite infectivity, suggesting that at end of the season, spores were better able to pass from the hosts gut into its haemolymph (Figure 6.6). In my laboratory study, parasites collected at different dates are exposed to the same standard host genotypes, whereas in the field, both host and parasite populations are likely to be changing, because hosts are likely to be experiencing parasite-mediated selection (DUFFY *et al.* 2009; DUNCAN and LITTLE 2007; DUNCAN *et al.* 2006). Indeed, Duncan *et al.* (2006) demonstrated that *D. magna* genotypes collected at the end of a *P. ramosa* epidemic were significantly more resistant than those collected at the beginning. Therefore, my experiment reveals parasite evolution in the laboratory, whereas in the field, I am witnessing the results of host-parasite coevolution.

I have field and corroborated laboratory evidence that a parasite population evolved greater infectivity and growth rate over the course of a single season. Further, I

find that these important parasite fitness traits are strongly negatively correlated with host abundance. Theory on the relationship between density and virulence predicts that high availability of susceptible hosts should favour *high* transmission (and virulence) (BULL 1994; DAY and GANDON 2007; EWALD 1993). However, this theory was developed for direct horizontal transmission of parasite propagules, whereas *P. ramosa* spores can remain in pond sediment for extended periods of time (years) before they infect a new host (see DECAESTECKER *et al.* 2004). (BONHOEFFER *et al.* 1996) argue that for parasites with long-living propagules (*e.g. P. ramosa*), increased host availability should only select for higher virulence and transmission (until equilibrium is reached) when the death rate of the host is low compared to the death rate of the parasite propagules. The fact that *P. ramosa* spores remain infectious for much longer than a *Daphnia*'s lifespan may therefore be why I observe a negative association between host abundance and parasite transmission potential.

Much of the theory investigating the evolution of parasite infection traits assumes that parasite virulence is associated with within-host growth, an assumption that is not universally supported (see ALIZON *et al.* 2009); Table 6.2). Indeed, a previous study of a different *Daphnia-Pasteuria* population found the fast growing parasites can be associated with low virulence (LITTLE *et al.* 2008), and in this study, I found no relationship between parasite growth and mortality (although a more powerful experiment is required to adequately test this). In the absence of a relationship between parasite growth and virulence, perhaps rare hosts favour infectious parasites that grow to a high density. After all, infection success is dose dependent in many host-parasite systems (BRUNNER *et al.* 2005; DE ROODE *et al.* 2007; OSNAS and LIVELY 2004), including *Daphnia-Pasteuria* (BEN-AMI *et al.* 2008b; EBERT *et al.* 2000; REGOES *et al.*

2002), so hosts from later in the season may require exposure to more parasite spores for infection to manifest. This effect would be amplified when the host population is simultaneously evolving resistance to infection (*i.e.* when there is coevolution), because the few hosts that are left at the end of the epidemic will have a higher mean resistance (*e.g.* DUNCAN *et al.* 2006).

Studies linking shifts in host abundance to parasite evolution on the same timescale are lacking, especially in the wild (see DYE and DAVIES 1990; MCCOY *et al.* 2005), and yet they are essential for us to better understand the role of virulence in host-parasite coevolution. My study demonstrates evolution in three parasite infection traits: infectivity, within-host growth and parasite ability to elicit a host cellular response all increase over the course of an epidemic, and these increases are associated with a decline in host abundance.

CHAPTER 7

General discussion

THE FITNESS CONSEQUENCES OF HOST IMMUNE DEFENCES FOR BOTH HOST AND PARASITE

Hosts exhibit abundant variation for both immune activity and ability to resist parasitic infection, leading evolutionary ecologists to examine the costs and benefits, in terms of host fitness, of different host immune strategies, as well as to question how these strategies relate to other life history traits (GRAHAM *et al.* 2011; ROLFF and SIVA-JOTHY 2003; SHELDON and VERHULST 1996). Some have gone on to assume that hosts with the highest immune activity (*e.g.* the greatest number of circulating haemocytes or the highest antibody titre) are those with the highest immunocompetence and the greatest fitness potential in the face of parasites (*e.g.* NUNN *et al.* 2000). This thesis provides a compelling example of how this assumption can mislead.

Daphnia magna that mount the strongest cellular response following exposure to *Pasteuria ramosa* were *most* likely to suffer parasitism in the future, and therefore had low fitness relative to their healthy counterparts. This was true of two *Daphnia-Pasteuria* populations: one in southern Germany and the other in Scotland, and was robust to both genetic variation in both host and parasite (Chapter 2, Chapter 4), and environmental variation in terms of initial parasite dose (Chapter 3). Also, already infected *Daphnia* had a significantly higher number of haemocytes than their healthy counterparts, both in the laboratory and in the wild (Chapter 5, Chapter 6). Collectively, these findings emphasize the fact that an immune response is a host's reaction to exposure to an infectious agent and that immune responses can be a consequence of infection as well as a cause of resistance (GRAHAM *et al.* 2011; READ

and ALLEN 2000; VINEY *et al.* 2005). In addition, the *Daphnia-Pasteuria* model used in this thesis is a naturally coevolving host-parasite system, and any infections occurred *via* the natural route (the host gut).

Host immune systems have been selected to (1) recognise infectious agents; (2) eliminate these agents; and (3) mend damage caused by these agents. But what makes a response an immune response? I argue that an immune response is a host's reaction to an infectious agent, although mis-directed immune responses are also known to mistakenly respond to host tissue (autoimmunity) or to benign non-infectious agents such as food, (allergy). From this viewpoint, immune responses can be a consequence of infection as well as a cause of resistance (GRAHAM *et al.* 2011; READ and ALLEN 2000; VINEY *et al.* 2005). *Daphnia magna*'s cellular response to *P. ramosa* does not lead to resistance to infection, and the potential role of the cellular response in resisting other parasites is, as yet, unknown. In Chapter 3, I examine the possibility that the cellular response may limit the fitness impact of infection on the host (*i.e.* minimize virulence); wound repair would be a possible mechanism for this. If this were the case, one would expect a positive association between haemocyte number and host fitness in infected hosts (measured as host offspring production). I found no evidence for such a relationship. This finding does not reject the hypothesis that *Daphnia* haemocytes are involved in wound repair, but it does suggest that wound repair has a minimal effect on fitness when *Daphnia* are infected with *P. ramosa*.

Throughout this thesis I have performed studies that have measured the increase in the number of haemocytes circulating in the *Daphnia* haemolymph following exposure to *P. ramosa.* It is important to note that while haemocyte number is closely associated with resistance to parasites in other invertebrates (ATAEV and COUSTAU

1999; ESLIN and PRÉVOST 1998; KRAAIJEVELD *et al.* 2001), there are limitations in measuring haemocyte number alone. Invertebrate haemocytes are known to produce potent cytotoxins including reactive oxygen and nitrogen species (CARTON and NAPPI 2001; NAPPI and OTTAVIANI 2000), and interact with other humoral components of the innate immune system (ELROD-ERICKSON *et al.* 2000). Haemocytes are also essential for haemolymph clotting, the principal mechanism of wound repair in invertebrates (IWANGA 2002) but see (THEOPOLD *et al.* 2004) and (STRAND 2008). A better understanding of how haemocyte function, for example the production of immune cytotoxins, varies across natural populations will greatly contribute to our knowledge of the role of host immune functions in the (co)evolution of hosts and their parasites.

Reconciling the broad nature of invertebrate immune recognition with genetic specificity for infection phenotypes

For years, molecular immunologists have found that invertebrate immune systems can only distinguish between broad classes of infectious agents (see HOFFMANN and REICHHART 2002). Exposure to different infectious agents causes the up-regulation of different immunological pathways: Gram-positive bacteria and fungi activate the Toll pathway and Gram-negative bacteria activate the Imd pathway (FERRANDON *et al.* 2003; HOFFMANN and REICHHART 2002; LEMAITRE *et al.* 1997; MEDZHITOV and JANEWAY 2000). However, evolutionary ecologists have found that a host's infection status after parasite exposure (infected or not) depends on the specific combination of host and parasite genotypes. This is referred to as genetic specificity (CARIUS *et al.* 2001; SCHMID-HEMPEL and REBER 2004) but see (LAMBRECHTS 2010), and suggests

invertebrate immune defences can distinguish between individual parasite genotypes. Studies linking measures of host immune activity with genetic specificity are, however, lacking (*c.f.* RIDDELL *et al.* 2009).

In Chapter 4, I found that that the *Daphnia* cellular response does *not* play a role in the phenomenon of genetic specificity: hosts mounted a cellular response to any spores that successfully breached the gut barrier defence, irrespective of parasite genotype. It is therefore likely that genetic specificity for infection occurred before the parasite entered the host's haemocoel, probably at the gut epithelium. Yet, while some have commented on the importance of barrier epithelia as a first line of defence against microparasites (see HOFFMANN and REICHHART 2002), the majority of studies examining invertebrate immune responses have injected the infectious agent directly into the host haemocoel, thereby bypassing this barrier defence (MORET and SCHMID-HEMPEL 2000; SADD and SIVA-JOTHY 2006; SCHWARTZ and KOELLA 2004). The cuticle or gut epithelia are not just simple walls that parasites need to overcome; they likely harbour a number of receptors able to recognise and block some parasite genotypes, but not others. This may explain the discrepancy between the genetic specificity observed at the whole-organism level and the broad nature of immune defences observed at the molecular level.

Findings from studies of *Drosophila*-parasitoid interactions are consistent with the hypothesis that genetic specificity manifests at the host epithelium. As discussed earlier, parasitoids inject their eggs directly into the host's haemocoel, naturally bypassing barrier defences, and *Drosophila*-parasitoid interactions are not explained by genetic specificity; both resistance and infectivity are instead graded traits (see KRAAIJEVELD and GODFRAY 1999 for a review). The ability of the Hymenopteran

parasitoid *Asobara tabida* to survive encapsulation from *Drosophila melanogaster* increases from North to South (KRAAIJEVELD and VAN ALPHEN 1994), and, while this ability does also depend on the identity of the host genotype, there is no host genotype-by-parasite genotype interaction (KRAAIJEVELD and GODFRAY 1999).

The collected data in this thesis support the two-stage heuristic model developed in Chapter 2 (see also AULD *et al.* 2010), where stage one involves the successful passage of the parasite from the host's gut into its haemolymph (penetrating a host barrier defence), and stage two involves the parasite withstanding, avoiding or counteracting immunological defences in the host's haemolymph. However, in order to effectively test this two-stage model, one needs to experimentally breach the host barrier defences and then examine both host immune responses and infection outcome (in terms of whether an infection occurs and the severity of the infection). I predict that, when injected, at least some non-infective parasite genotypes would successfully infect (and achieve fitness) in otherwise resistant hosts. If this model holds true, the exact point where an infection fails could have profound implications for both host and parasite fitness.

The fitness implications of the two-stage model for host and parasite

The two-stage model may explain when hosts pay a certain fitness costs for resisting parasitism. Such costs fall into two categories. Firstly, there are costs of maintaining immune defences, which occur even when parasites are absent. These manifest as negative genetic covariance between immunity and other fitness-related trait(s). For example *Drosophila melanogaster* that have been selected for increased resistance to

the parasitoid, *Asobara tabida,* are less able to compete for food when at the larval stage (KRAAIJEVELD and GODFRAY 1997), and *Plodia interpunctella* moths selected for increased resistance to the virus have a longer development time (BOOTS and BEGON 1993). Secondly, there are costs of mobilising an immune response. For example, *D. melanogaster* suffer reduced fecundity when they successfully resist *Tubulinosema kingi* (VIJENDRAVARMA *et al.* 2008), and the beetle *Tribolium castaneum* has a longer development time and suffers reduced survival when exposed to heat-killed bacteria (ROTH and KURTZ 2008). Most of the studies that reveal costs of immune mobilization do so with hosts experimentally injected with a pathogen or immune stimulant or where the host is exposed to a parasitoid (which itself overcomes barrier defences). Where the first stage of infection has been artificially bypassed by injection, we may have (in some cases) been exposing mobilisation costs that would not ordinarily occur in nature.

Mobilisation costs of resistance in the *Daphnia-Pasteuria* system are rare (LABBÉ *et al.* 2010), perhaps because in most cases where infection is unsuccessful, the parasite has not passed the gut epithelium and elicited an immune response in the host (*i.e.,* it has failed at stage one of the infection process). The only study to detect a mobilisation cost of immunity in *D. magna* (where resistant *Daphnia* had increased mortality: (LITTLE and KILLICK 2007) did so in a host-parasite genotype combination where a where the parasite has since been shown to elicit a cellular response in the host (host GG4 and parasite Sp1: Chapter 2, Chapter 3, Chapter 4; see also AULD *et al.* 2010), suggesting that the parasite spores passed from host gut to haemolymph, *i.e.,* the parasite was successful at stage one of infection but failed at stage two. My studies did not uncover a mobilisation cost of resistance in terms of mortality, probably because mortality was very low; however, in Chapter 4, I found in host-parasite genotype

combinations where cellular responses were elicited (and infections were documented), there was elevated fecundity in uninfected hosts, *i.e.* the suggestion of fecundity compensation (documented in a different *Daphnia*-microparasite system (CHADWICK and LITTLE 2005). These findings suggest that host life-history shifts following parasite exposure (including fitness costs for immune activation) only occur when the parasite breaches host barrier defences. In many cases, the haemolymph-based host immune system may not be subject to parasite-mediated selection, because it does not come into contact with the parasite. This does, however, require further investigation.

Also, a parasite's failure to overcome host barrier defences may lead to very different fitness consequences (for the parasite) than failure to infect once inside the host's haemocoel (see Chapter 4). A parasite that penetrates a cuticle or gut epithelium and is destroyed by haemolymph-based defences is dead; it will never achieve fitness. Conversely, a parasite that fails to penetrate the initial barrier may not be killed. *Pasteuria ramosa* spores have been selected to remain infectious through tough environmental conditions, including desiccation (DECAESTECKER *et al.* 2007; DECAESTECKER *et al.* 2004), so if they fail to infect a host, perhaps these hardy spores pass through the gut unharmed and will have the opportunity to infect a different in the future. If so, parasite fitness would be delayed and not terminated.

Other studies have found non-infecting parasites are killed by their invertebrate hosts: *Metschnikowia bicuspidata,* a yeast-like fungal parasite of *Daphnia* suffers high mortality in the host gut if it fails to successfully infect (HALL *et al.* 2009), as do *Microphallus* trematodes, parasites of the snail *Potamopyrgus antipodarum* (KING *et al.* in press); however, these parasites are not as hardy as *P. ramosa* spores. The ability to pass through the host gut unharmed would have interesting consequences for both

evolutionary and epidemiological dynamics of disease. Firstly, it could maintain diversity in the parasite population which would increase the likelihood of future infections occurring in the host population (GANZ and EBERT 2010). Second, this abundant genetic variation would increase the parasite population's evolutionary potential, allowing more rapid adaptation following a shift in biotic or abiotic conditions. A parasite's ability to pass through a host unharmed may also depend on host genotype, parasite genotype or the combination of both, *i.e.,* it may depend on genetic specificity. This remains to be investigated.

THE IMPORTANCE OF STUDYING (CO)EVOLUTION IN THE FIELD: OPPORTUNITIES WITH THE *DAPHNIA-PASTEURIA* SYSTEM

Pasteuria ramosa applies selection that results in evolution in a natural *D. magna* population: the genotypic composition of the host population changes over the course of the season, and hosts are more resistant to *P. ramosa* infection after an epidemic than before (DUNCAN and LITTLE 2007; DUNCAN *et al.* 2006). In Chapter 6, I find that the parasite population also evolves over the course of the season: *P. ramosa* spores collected after an epidemic have a higher infectivity and greater within-host growth than *P. ramosa* spores collected early in the season; they also elicit a stronger cellular response in the host. Further, this evolution in parasite fitness traits is associated with a decline in host abundance (Chapter 6).

In all of these studies, infection phenotypes were documented in the wild and then empirically examined under controlled laboratory conditions: in Duncan *et al.*

(2006) and Duncan and Little (2007), wild *Daphnia* genotypes (clones) were collected from the field and then their resistance to *P. ramosa* was later determined by exposing them to a fixed dose of parasite spores under standard laboratory conditions; and in my study (Chapter 6), *P. ramosa* spores were collected from the field and a fixed dose was exposed to standard host clones and infection traits were recorded, again in the laboratory (Chapter 6).

Most real-time studies of coevolution are conducted with laboratory microbial organisms with short generation times (*e.g.* MORGAN *et al.* 2005; PATERSON *et al.* 2010; RAINEY 2004), whereas studies in wild host-parasite systems are usually not able to document coevolution in real-time. Instead, these field studies look for the signatures of coevolution, either in spatial patterns of host resistance and parasite infectivity (*e.g.* THRALL and BURDON 2003), on in the host or parasite genomes (MCCOY *et al.* 2003). The *Daphnia-Pasteuria* system provides a prime opportunity to study host-parasite coevolution in real-time. It would be possible to bring wild *Daphnia* and *Pasteuria* into the laboratory and determine: (a) in which host genotypes was the parasite most prevalent and had the highest spore densities; (b) whether changes in parasite infection traits (infectivity, within-host growth and virulence) were associated with host genotype; and therefore (c) whether there was coevolution between host and parasite *in the field.* (LYTHGOE and READ 1998) wrote: "Are natural populations like ducks on a river, with calm stasis on the surface being maintained by frantic paddling beneath the surface at the genetic level?" Perhaps with the *Daphnia-Pasteuria* system, we can better understand that frantic paddling.

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APPENDIX: PUBLISHED PAPERS

PROCEEDINGS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Genetic variation in the cellular response of *Daphnia magna* (Crustacea: Cladocera) to its bacterial parasite

Stuart K. J. R. Auld, Jennifer A. Scholefield and Tom J. Little

Proc. R. Soc. B 2010 **277**, 3291-3297 first published online 9 June 2010
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Genetic variation in the cellular response of *Daphnia magna* (Crustacea: Cladocera) to its bacterial parasite

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Linking measures of immune function with infection, and ultimately, host and parasite fitness is a major goal in the field of ecological immunology. In this study, we tested for the presence and timing of a cellular immune response in the crustacean *Daphnia magna* following exposure to its sterilizing endoparasite *Pasteuria ramosa*. We found that *D. magna* possesses two cell types circulating in the haemolymph: a spherical one, which we call a granulocyte and an irregular-shaped amoeboid cell first described by Metchnikoff over 125 years ago. *Daphnia magna* mounts a strong cellular response (of the amoeboid cells) just a few hours after parasite exposure. We further tested for, and found, considerable genetic variation for the magnitude of this cellular response. These data fostered a heuristic model of resistance in this naturally coevolving host–parasite interaction. Specifically, the strongest cellular responses were found in the most susceptible hosts, indicating resistance is not always borne from a response that destroys invading parasites, but rather stems from mechanisms that prevent their initial entry. Thus, *D. magna* may have a two-stage defence—a genetically determined barrier to parasite establishment and a cellular response once establishment has begun.

Keywords: invertebrate immunity; haemocytes; host–parasite coevolution; resistance; *Daphnia*, *Pasteuria*

1. INTRODUCTION

Parasites often impose substantial costs on their hosts, as evidenced both by the severe effects they can have on individuals, and in the impact they may have on host population sizes (Van Alfen *et al.* 1975; Hudson *et al.* 1998; Duncan & Little 2007). Host defence mechanisms, therefore make a key contribution to organismal fitness and genetic variation for these mechanisms may contribute to host evolution in the face of parasitism. The first line of defence for the invertebrate host often consists of the barrier defences of the cuticle or more complex defences of the gut epithelium (Artis 2008). After these come the haemolymph-based immune defences, for example, phagocytic haemocytes, antimicrobial peptides or lysozymes (Hoffmann 2003; Mydlarz *et al.* 2006). Much of our understanding of invertebrate immunity is built on studies of insect–parasite systems, although there are notable exceptions (Mydlarz *et al.* 2006). We argue the importance of strengthening our knowledge of invertebrate immunity beyond the insects, as well as the need to develop deep understanding of the interplay between naturally coevolving antagonists.

One of the goals of ecological immunology is to determine the role immunological mechanisms play in mediating variation in fitness when organisms are exposed to parasites. To address the function that immune responses have in determining infection outcomes and, ultimately, the fitness consequences of infection (or self-harm owing to immunopathology), it is necessary to measure how immune effector systems vary under genetic and environmental variation. However, many studies aiming to elucidate immune mechanisms have done so in the absence of pathogens, under controlled laboratory conditions and in homogeneous, inbred genetic backgrounds. Thus, while providing the necessary mechanistic backbone for studying the immune function, this approach does not address variation in natural populations (Little *et al.* 2005). However, a considerable body of evidence suggests that the impact of genetic and environmental variation on infection is substantial (Mydlarz *et al.* 2006; Lazzaro & Little 2009), and it is thus difficult to extrapolate from laboratory measures of immune responsiveness to variation in fitness (Viney *et al.* 2005).

Here, we tested for a cellular immune response in a naturally coevolving host–parasite model: the aquatic crustacean, *Daphnia magna* and its sterilizing bacterial endoparasite, *Pasteuria ramosa*. The fitness consequences, for example, host sterilization or mortality due to *P. ramosa* infection have been extensively studied under genetic and environmental variation (Mitchell *et al.* 2005; Duncan *et al.* 2006; Vale *et al.* 2008; Vale & Little 2009), but the mechanisms of resistance have received less attention in this system (Mucklow & Ebert 2003; Mucklow *et al.* 2004; Labbe *et al.* 2009). Circulating haemocytes are an important anti-parasite defence in many invertebrates (Ataev & Coustau 1999; Elrod-Erickson *et al.* 2000; Kraaijeveld *et al.* 2001; Canesi *et al.* 2002; Cotter *et al.* 2004), and have been found in *D. magna* (Metchnikoff 1884). They are central to the innate immune system, being involved in phagocytosis and encapsulation; they are also vehicles for other immune

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functions, e.g. the generation of reactive oxygen and nitrogen species, as well as antimicrobial peptides and phenoloxidase (Strand 2008). For these reasons, we chose them as the immune marker for this study. Both the induction of a cellular response and its magnitude are likely to contribute to host fitness when the host is in the presence of parasites.

This study also examines how the magnitude of cellular response varies across multiple host genotypes. By embracing host genetic variation, we hope to gain further insight into how parasitism could influence host genetic structure, and ultimately, host evolution. We also test how infection outcome differs across host genotypes, allowing us to link our measures of cellular response with susceptibility. Finally, we sought to determine whether it is the mere presence of parasite spores in the gut, or the process of spores moving from the gut to haemolymph that elicits a cellular response in the host.

2. MATERIAL AND METHODS

(a) *Host and parasite organisms*

Daphnia magna is a freshwater crustacean of shallow, eutrophic ponds. It reproduces by cyclical parthenogenesis, where apomictic parthenogenesis is the main reproductive mechanism, but bouts of sexual reproduction occur in the presence of specific cues (Carvalho & Hughes 1983; Hobaek & Larsson 1990; Slarsarczyk *et al.* 2005). By keeping *D. magna* in the absence of sexual cues, purely clonal lines can be maintained in the laboratory.

Pasteuria ramosa is a spore-forming, bacterial endoparasite, obligate to *D. magna*. It is transmitted horizontally from dead, infected hosts (Ebert *et al.* 1996), and is believed to infect via the gut and proliferate in the host's haemolymph. Successful *P. ramosa* infections have a profound impact on host fitness, often causing complete host sterilization and premature death (Ebert *et al.* 1996).

Twelve of the 16 host genotypes used here were founded from a single animal, hatched from an ephippium (sexually produced resting egg) in the laboratory. Ephippia were from pond mud collected in Gaazerfeld, Germany in 1997. The other four genotypes (numbers 3, 4, 7 and 13) were also founded from single individuals, but these were collected as adults from Gaazerfeld in 1997 and have since been kept in a state of clonal reproduction. The *P. ramosa* isolate originated from a single infected *D. magna* from that same pond (Carius *et al.* 2001), and has been used in a variety of experiments since that time. The *P. ramosa* spore solution used here was made by homogenizing previously infected hosts with ddH_2O.

(b) *Experimental set-up*

Independent replicates for each *D. magna* genotype were maintained for three generations to minimize variation in condition. Animals were kept in jars containing 200 ml of artificial medium (Kluttgen *et al.* 1994) modified using one-twentieth of the recommended SeO_2 concentration (Ebert *et al.* 1998) and fed 5.0 ABS *Chlorella vulgaris* algal cells per day (ABS is the optical absorbance of 650 nm white light by the *Chlorella* culture). Their medium was refreshed three times per week. There were five *Daphnia* per jar and jars were incubated at 20°C on a 12L : 12D light cycle. The second-clutch neonates from the third generation were used in each of the four experiments.

The first experiment examined host cellular response in four host clones or genotypes. For this four-genotype cell experiment, replicates were allocated to one of two parasite treatments: non-exposed or parasite-exposed. Parasite treatment lasted for 2 h, 4 h, 6 h or 8 h. Thus, there were six replicates per genotype, per parasite treatment, per time treatment. The second and third experiments both studied 16 genotypes: the second experiment examined host cellular response and the third experiment measured infection outcome. Like the previous four-genotype cell experiment, replicates were allocated to one of two parasite treatments (non-exposed or parasite-exposed), however all replicates were exposed for the same amount of time: 5 h. There were six and twelve replicates per parasite treatment, per genotype for the second and third experiment, respectively. Finally, a fourth experiment used one genotype (genotype 4 from the previous experiment) to test for the presence of a cellular response when the host was exposed to killed (non-infective) spores or live (infective) spores. Spores were killed by heating them in a water bath at 95°C for 30 min. Replicates were allocated to three treatments: non-exposed and parasite-exposed, and exposed to killed parasites. There were eight replicates per treatment.

Parasite exposures were carried out as follows. When at least three out of five of the *Daphnia* in a replicate had deposited eggs in their brood chamber, the replicate was exposed to its parasite treatment. The five *Daphnia* of the replicate were placed together in a well of a 24-well cell plate (Costar, Corning Inc., NY, USA). Parasite-exposed replicates received 50 000 *P. ramosa* spores from the pre-prepared solution. Non-exposed control replicates received the same concentration of uninfected *D. magna* homogenized in ddH_2O.

(c) *Haemocyte collection and counting*

After parasite treatment, five *Daphnia* from each replicate were placed in a cell extraction chamber containing 4.0 µl of ice-cold anticoagulant buffer (98 mM NaOH, 186 mM NaCl, 17 mM EDTA and 41 mM citric acid, pH adjusted to 4.5: Lavine *et al.* 2005). A 25-guage needle (BD Microlance, Drogheda, Ireland) was used to pierce the *Daphnia* heart, causing haemolymph to pool into the medium. The *Daphnia* were then removed and the haemolymph solution was mixed thoroughly using a pipette. Four microlitres of the cell suspension were placed in a fertility counting chamber ($0.001\ mm^2 \times 0.100$ mm (depth); Hawksley, Lancing, Sussex, UK), and the number of amoeboid haemocytes was counted (figure 1). The number of granulocytes did not vary between treatments in any of the cell experiments and are not discussed further. Haemocyte counts were converted to number of cells per microlitre of haemolymph–buffer solution.

(d) *Life-history assays*

After parasite treatment, one of the five *Daphnia* from each replicate of the 16-clone life-history experiment was randomly selected and kept individually in 60 ml of artificial medium and fed 1.0 ABS *C. vulgaris* cells per day. Their medium was refreshed three times per week, or after the *Daphnia* had a clutch of offspring, and jars were incubated at 20°C on a 12L : 12D light cycle. Jars were checked daily for clutches and the number of offspring was recorded at each clutch. From day 25 post-parasite exposure, hosts were examined for symptoms of *P. ramosa* infection. Symptoms include cessation of reproduction, absence of ovaries

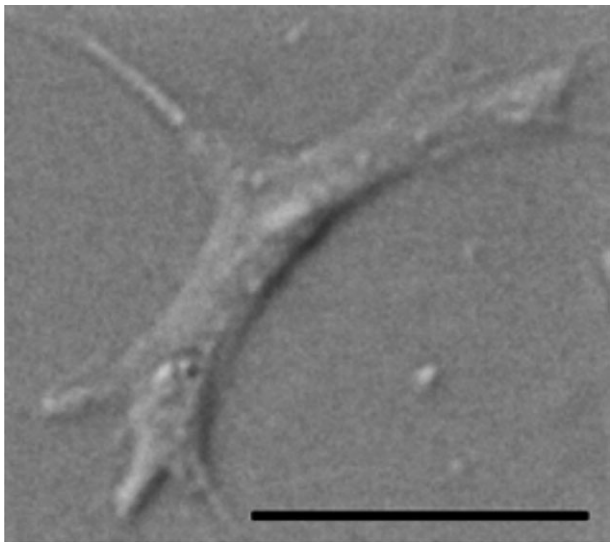

Figure 1. Differential interference contrast image of an amoeboid haemocyte from *D. magna*. Scale bar, 5 μm.

and bacterial growth in the haemolymph. The experiment ran for 32 days.

(e) *Statistical analyses*

Data were analysed using R (Ihaka & Gentleman 1996; R Development Core Team 2005). To achieve normality of distribution in the data, haemocyte counts were log-transformed for the four genotype and 16-genotype cell experiments and square-root transformed for the killed parasite cell experiment. For the four-genotype cell experiment, we tested the fixed effects of host genotype, parasite treatment and exposure time, as well as all interaction terms. For the 16-genotype cell experiment, we tested the fixed effects of host genotype and parasite exposure along with their interaction. Welch's two sample *t*-tests were performed *post hoc* on the 16-genotype cell data to test for the presence of a significant cellular response in each of the host genotypes, and the results were corrected for multiple comparisons (Holm 1979). For the killed parasite experiment, we tested for differences between parasite–exposure treatments.

We report the full statistical models for both the four-genotype and 16-genotype cell data, along with the proportion of the variance explained by each of the terms in the full model. Variance proportions were calculated by dividing the sequential sum of squares for each term by the total sum of squares for the model. We then multiplied these proportions by 100 to find the percentage variance explained by each term.

3. RESULTS

(a) *Four-genotype cell experiment*

Haemocyte counts were obtained from 240 *Daphnia* from 48 jars. Averaging across all genotypes, mean circulating haemocyte number per microlitre from the *P. ramosa*-exposed replicates was 599 ± 80 ($n = 24$), whereas control replicates had a mean of 196 ± 11 circulating haemocytes ($n = 24$). However, the magnitude of the parasite-induced cellular response depended on the identity of the host genotype: i.e. there was a parasite exposure by host genotype interaction (figure 2 and table 1). When genotype is coded as a random effect, parasite exposure remains significant ($F_{1,3} = 15.26$, $p < 0.05$), and a model with the parasite exposure-by-genotype effect explained significantly more variation than did a model without the interaction term ($\chi^2 = 4.60$, d.f. $= 1$, $p < 0.05$).

(b) *16-genotype cell experiment*

Haemocyte counts were obtained from 960 *Daphnia* from 192 jars. As before, a cellular response followed *P. ramosa* exposure, with a mean per microlitre haemocyte count that was highly consistent with the previous experiment: 614 ± 50 cells for *P. ramosa*-exposed replicates ($n = 96$) and 208 ± 17 haemocytes per microlitre for control jars ($n = 96$). Basal haemocyte counts differed across host genotypes ($F_{15,80} = 4.49$, $p < 0.001$); and, there was also considerable genetic variation in the magnitude of cellular response, varying between a one and ninefold increase in haemocyte number depending on the identity of the host genotype (figure 3). Statistically, this appears as a strong parasite exposure by host genotype interaction (table 2). The three host genotypes that mounted the strongest cellular response were the three genotypes that suffered infection from *P. ramosa* (figure 3). Again, the parasite treatment remains significant with genotype as a random effect ($F_{1,15} = 27.76$, $p < 0.0001$), and the parasite exposure-by-genotype effect explained significantly more variation than did a model without the interaction term ($\chi^2 = 32.86$, d.f. $= 1$, $p < 0.0001$).

Post hoc tests revealed a significant cellular response, i.e. that the number of circulating haemocytes was greater in exposed versus unexposed in the following five host genotypes: 3, 4, 17, 20 and 22 (figure 3). This was after the data were corrected using the sequential Bonferroni adjustment (Holm 1979). Of these five responding genotypes, three suffered infection from *P. ramosa* (3, 4 and 17).

(c) *16-genotype life-history experiment*

Successful infection was recorded in three of the 16 genotypes, where infection with *P. ramosa* caused a substantial reduction in the number of offspring produced by the *Daphnia*. Of replicates from the parasite-exposed treatment, uninfected hosts had 48.05 ± 0.78 offspring, whereas infected hosts had 32.21 ± 1.15 offspring ($t = 11.35$, d.f. $= 47.61$, $p < 0.0001$).

(d) *Killed parasite cell experiment*

Haemocyte counts were obtained from 120 *Daphnia* from 24 jars. The strongest cellular response followed exposure to live parasite spores, with a mean haemocyte count of 584 ± 83 haemocytes per microlitre for live *P. ramosa*-exposed jars ($n = 8$) and 65 ± 13 for control jars ($n = 8$). There was also a smaller but significant cellular response from jars exposed to heat-treated *P. ramosa* spores: 238 ± 30 haemocytes ($n = 8$). *Post hoc* tests revealed that haemocyte counts from all treatments were significantly different from each other (Tukey's HSD, $p < 0.05$). Only jars exposed to live *P. ramosa* spores went on to develop infection (data not shown).

4. DISCUSSION

Just hours after exposure to the bacterial parasite *P. ramosa*, there was a large increase in the number of amoeboid cells circulating in the haemolymph of

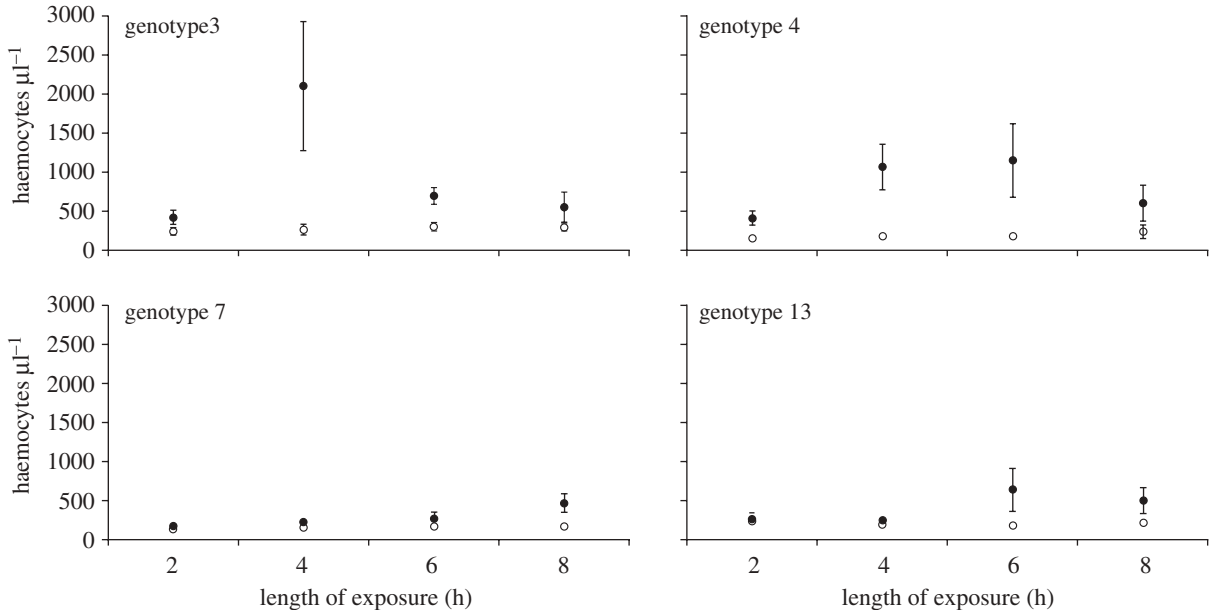

Figure 2. Haemocyte counts per host in *P. ramosa*-exposed and control *D. magna* (filled and open symbols, respectively; $n = 6$ and each replicate consists of five *Daphnia*). Error bars are 1 s.e.m. See table 1 for statistical details.

Table 1. Summary of analysis of the number of circulating haemocytes in an experiment involving four host genotypes of *D. magna*. The effects tested were parasite (exposed or not), time post-exposure and host genotype.

number of haemocytes	d.f.	*F*	*p*	% var [a]
time	3	2.18	0.09	2.19
parasite	1	61.31	<0.0001	20.57
genotype	3	11.13	<0.0001	11.2
time × parasite	3	1.82	0.14	1.84
time × genotype	9	1.09	0.37	3.29
parasite × genotype	3	4.02	<0.01	4.05
time × parasite × genotype	9	1.05	0.40	3.18
error	160			53.69

[a]Percentage of the total variance explained by each term in the full model.

D. magna. These data also revealed very large differences in cellular response between host genotypes, ranging from no increase to a greater-than ninefold increase in cell number (figures 2 and 3). Basal (uninduced) haemocyte counts did differ across host genotypes, but these differences did not predict the likelihood of becoming infected. This differs from the finding that *Drosophila melanogaster* with a greater basal haemocyte level were more resistant to parasitoid infection (Kraaijeveld *et al.* 2001). Non-infective parasite spores (i.e. those we heat-killed prior to exposure) elicited a small increase in the number of circulating haemocytes, suggesting that the presence of parasite material in the gut may trigger weak immune reactions; perhaps bacterial ligands are penetrating the gut mucosa and triggering an immune response (Raz 2010). However, data from the killed-spore experiment clearly show that live infective spores induce a much stronger cellular response.

This cellular response is possibly the host immune response that the parasite encounters when it passes from the host gut into its body, supporting very early work showing *D. magna* mounts a cellular response to a yeast-like infection (Metchnikoff 1884). Immune function and immunity, however, are clearly *not* one and the same: the largest increase in cell numbers was seen in the host genotypes that were susceptible to the parasite (figure 3). Other studies of putative immune responses found no link between infection status and strength of the response (e.g. Mucklow *et al.* 2004). If the cellular immune response to *P. ramosa* depends upon the parasite spores passing the gut epithelium, complete resistance appears to be achieved by preventing that passage (as opposed to destroying parasites once they have gained access). A very strong cellular response thus appears to be indicative of a critical failure elsewhere in the host immune system (most likely in the gut epithelium), and it appears that the gut epithelium forms the main defence.

The *P. ramosa* infection process may be similar to that seen in *Pasteuria penetrans*, a sterilizing parasite that initiates infections by attaching to the heparin-binding domain and gelatine-binding domain proteins on the cuticle of *Meloidogyne* nematodes (Sayre & Starr 1985; Mohan *et al.* 2001; Schmidt *et al.* 2008). The external surface of the nematode encounters *P. penetrans* as it migrates through the soil, whereas *P. ramosa* is thought to be taken up as the *D. magna* filter feed where it then penetrates the gut. Aside from this difference in the location of infection, *P. ramosa* may similarly require

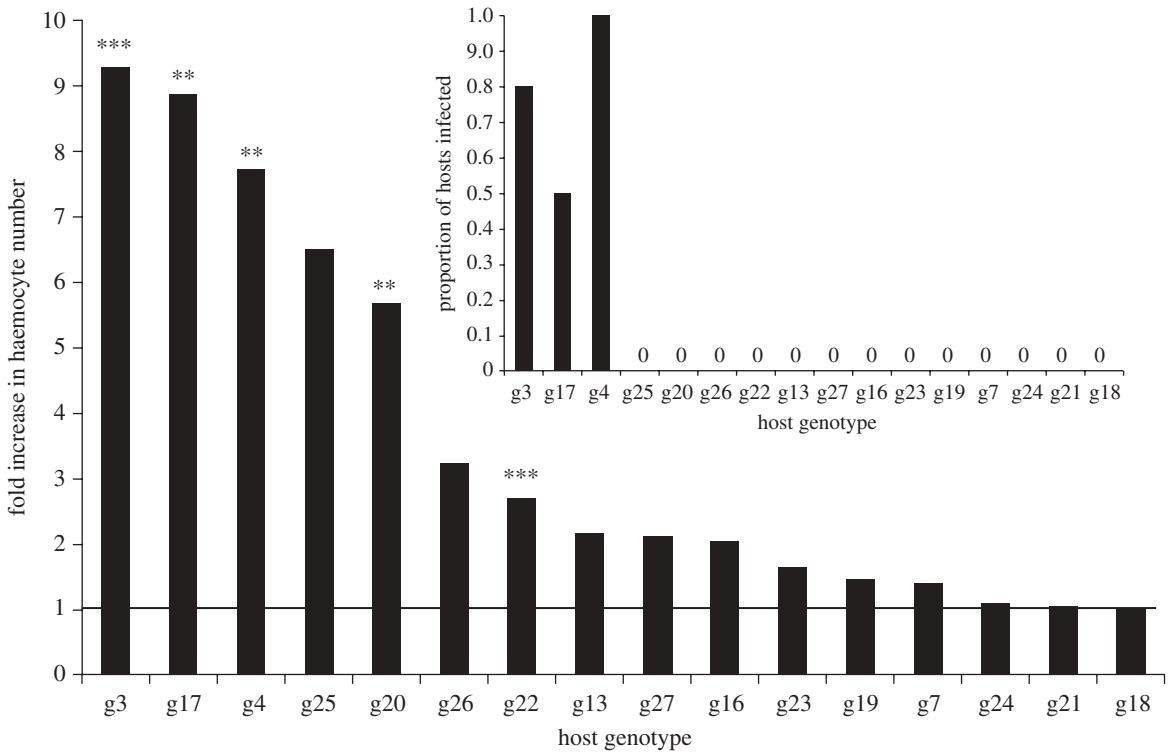

Figure 3. Fold induction of haemocyte numbers in *P. ramosa*-exposed *D. magna* ($n = 6$, each replicate consists of five *Daphnia*), relative to unexposed *D. magna* ($n = 6$, each replicate consists of five *Daphnia*). The bold line at $y = 1$ shows the uninduced (basal) level. The inset shows the proportion of individuals that became infected in *P. ramosa*-exposed treatments in each genotype ($n = 12$, each replicate consists of an individual *Daphnia*). Asterisks indicate if haemocyte numbers rose significantly (after sequential Bonferroni adjustment) above basal levels: ** $p < 0.01$, *** $p < 0.001$.

Table 2. Summary of analysis of the number of circulating haemocytes in an experiment involving 16 host genotypes of *D. magna*. The effects tested were parasite (exposed or not) and host genotype.

number of haemocytes	d.f.	*F*	*p*	% var[a]
parasite	1	157.29	<0.0001	28.53
genotype	15	9.72	<0.0001	26.67
parasite × genotype	15	5.67	<0.0001	15.54
error	160			29.26

[a]Percentage of the total variance explained by each term in the full model.

binding to epithelial proteins to initiate infection, and without this binding the infection process, subsequent cellular response will not occur. The probability of molecular binding to *D. magna* epithelial proteins appears to be subject to host genetic variation; or, there is variation in other gut-based defences. We propose that a lack of molecular matching explains cases of resistance, while a strong cellular response indicates a molecular genetic match that allows parasites to overcome gut defences. This heuristic model of a two-tiered defence is largely supported by the observation that the three susceptible host genotypes had the strongest cellular responses, while the majority of non-responding genotypes remained healthy (figure 3). Still, two host genotypes responded to parasite exposure but showed no signs of infection, which indicates that the cellular immune response may only play a limited role in resistance, if only a very small number of spores reach the haemolymph.

Previous work has modelled the genetics of infection as a two-stage process, with 'matching-allele' genetics for parasite detection, and 'gene-for-gene' genetics for parasite eradication (Agrawal & Lively 2003). *Daphnia magna*'s patterns of resistance and cellular responses to *P. ramosa* can be used to test such models. Thus, a desirable follow-up study to the present work comparing host genotypes would be experiments incorporating both host and parasite genetic variation (*sensu* Carius *et al.* 2001), as well as with parasites from other taxa, where a cellular response may successfully provide resistance. Studies of such genetic specificity and the cellular response would be the next step towards elucidating the immunological basis of invertebrate coevolutionary interactions.

A substantial body of work in invertebrate immunology has studied the response to opportunistic bacteria, generalist entomopathogens or chemical pathogen mimics (e.g. LPS); and there are considerable merits in measuring immune function in non-coevolved systems (Barnes & Siva-Jothy 2000), primarily that the parasite has not had the opportunity to evolve avoidance of host immune responses (Huxham *et al.* 1988; Barnes & Siva-Jothy 2000). By adopting such an approach one can better assess the generality of a host's immune function without the confounding influence of anti-parasite defence mechanisms. Conversely, our use of a naturally coevolving host–parasite combination means the cellular response we document reflects how invertebrates defend themselves against natural enemies. Indeed, outside of the well-studied interaction between mosquitoes and *Plasmodium* parasites, we have little understanding of

the invertebrate immune response to coevolving biological enemies. Thus, in the study of invertebrate immunity, our work is a rare example of the (putative) immune response and genetic variation for that response, against a natural parasite.

It is now widely acknowledged that a stronger immune response does not necessarily lead to higher fitness—the relationship between host fitness and both size of immune response and parasite burden may not be linear (Adamo 2004; Viney *et al.* 2005; Stjernman *et al.* 2008). Our work is a compelling example of this point: had we measured only haemocyte responsiveness without assessing infection probabilities (and hence fitness), a misleading impression of which is the fittest genotype would have emerged. This argues against the practice (common in the early days of ecological immunology) of measuring immune parameters in isolation from infection biology. Moreover, the large differences in cellular response between host genotypes emphasizes the need to embrace genetic variation when studying immune function. Had we looked for a cellular response in just one host genotype, our results would very much depend on which genotype we studied. For example, a study of host genotype 3 would lead to opposite conclusions to a study of genotype 18. This makes clear the need to effectively link studies of immune function to studies of infection outcome in multiple host genotypes. That being so, the next stage is to investigate the role of parasite genetic variation: both how it modifies cellular response in different host genotypes, and how this links to infection outcome.

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ECOLOGICAL IMMUNOLOGY

Fitness consequences of immune responses: strengthening the empirical framework for ecoimmunology

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Summary

1. Ecoimmunologists aim to understand the costs, benefits, and net fitness consequences of different strategies for immune defense.

2. Measuring the fitness consequences of immune responses is difficult, partly because of complex relationships between host fitness and the within-host density of parasites and immunological cells or molecules. In particular, neither the strongest immune responses nor the lowest parasite densities necessarily maximize host fitness.

3. Here, we propose that ecoimmunologists should routinely endeavour to measure three intertwined parameters: host fitness, parasite density, and relevant immune responses. We further propose that analyses of relationships among these traits would benefit from the statistical machinery used for analyses of phenotypic plasticity and/or methods that are robust to the bi-directional causation inherent in host-parasite relationships. For example, analyses of how host fitness depends upon parasite density, which is an evolutionary ecological definition of tolerance, would benefit from these more robust methods.

4. Together, these steps promote rigorous quantification of the fitness consequences of immune responses. Such quantification is essential if ecoimmunologists are to decipher causes of immune polymorphism in nature and predict trajectories of natural selection on immune defense.

Key-words: bivariate statistics, *Daphnia*, evolutionary parasitology, immunocompetence, optimal immunity, random regression, resistance, tolerance

Introduction

Hosts vary greatly in the strength of their immune responses and their capacity to defend themselves against parasites. Ecoimmunologists shed light on this variation by characterizing optimal defense strategies in a world of life-history tradeoffs, unpredictable epidemics, polyparasitism, and genetic and environmental variation (Medley 2002; Rolff & Siva-Jothy 2003; Lazzaro & Little 2009; Sadd & Schmid-Hempel 2009). Accordingly, a basic requirement of empirical studies in ecoimmunology is to measure and interpret the fitness consequences of immune responses – in other words, to ascertain the impact of cellular or molecular responses to infection (hereafter, 'immune responses') upon the lifetime reproductive success (hereafter, 'fitness') of the responder. But this basic requirement poses serious challenges.

Ecoimmunologists increasingly appreciate that two 'shortcuts' to estimating the fitness consequences of immune responses must be avoided. The first is to count immunological cells or molecules and assume that hosts producing the most hemocytes or antibodies, for example, are the most fit (e.g. Nunn, Gittleman & Antonovics 2000 as critiqued by Read & Allen 2000). The second is to quantify parasite densities and assume that hosts bearing the most parasites are the least fit (e.g. see critique in Behnke, Barnard & Wakelin 1992). These shortcuts fail because the magnitude of an immune response does not always correlate positively with host fitness

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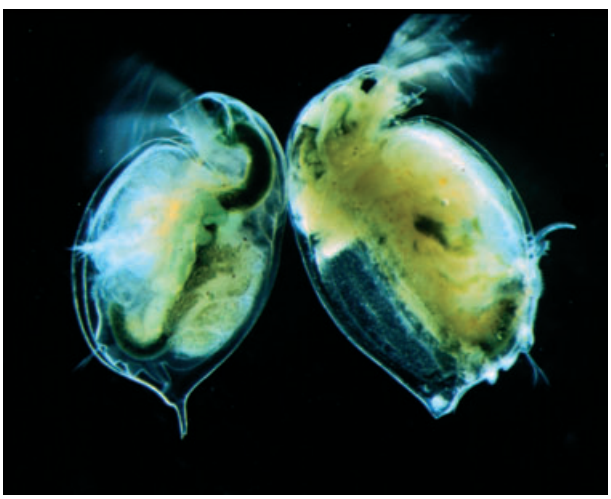
(Adamo 2004; Graham, Allen & Read 2005; Rolff & Siva-Jothy 2003, Sadd & Schmid-Hempel 2009, Viney, Riley & Buchanan 2005), and hosts that kill all of their parasites are not necessarily better off: host fitness may be maximal at some intermediate parasite density (Behnke, Barnard & Wakelin 1992; Viney, Riley & Buchanan 2005; Stjernman, Raberg & Nilsson 2008). As a result, the relationship between host fitness and parasite density – sometimes called tolerance by evolutionary ecologists – has received a lot of attention lately (Raberg, Sim & Read 2007; Ayres & Schneider 2008, 2009; Pagan, Alonso-Blanco & Garcia-Arenal 2009; Raberg, Graham & Read 2009); also see summary of controversy below.

Here, we aim to cement the view that ecoimmunologists should aim to quantify how host fitness is affected by both parasite density and immune response magnitude. Measuring this triad of traits offers the best opportunity to interpret ecological variation in immunity. We stress that each trait is likely to be the product of an interplay between host and parasite genes, which has important consequences for empirical practice and for inferring evolutionary outcomes. We propose that a combination of controlled experiments and statistical

Box 1. From evolutionary genetics to ecoimmunology in lab and field: *Daphnia magna–Pasteuria ramosa* as a 'model' system

Daphnia are small (~1–3 mm), ubiquitous freshwater crustaceans that have been the focus of a large and diverse literature, including toxicology, life-history, physiology, nutrition and parasitology. *Daphnia* were also the subject of pioneering work on invertebrate cellular immunology (Metchnikoff 1884), an area that has recently been revisited within the ecoimmunology framework (Auld, Scholefield & Little 2010) (Boxes 2 and 3). In the field, gathering epidemiological data is relatively straightforward because the clear carapace of *Daphnia* makes many infections easy to identify. In the photograph, the left *D. magna* is healthy (note embryos in the brood chamber), while the right *D. magna* is infected with the bacterium *Pasteuria ramosa,* which sterilizes hosts leading to an empty brood chamber (a clear indication of reduced host fitness). Epidemics are common and severe in this system, but highly variable in space and time (Stirnadel & Ebert 1997; Duncan, Mitchell & Little 2006; Lass & Ebert 2006; Duncan & Little 2007). With parasite density and indeed parasite fitness being further quantifiable because transmission spores are easily counted, the recommended triad of traits – host fitness, within-host parasite density, and immune response magnitude – are measurable.

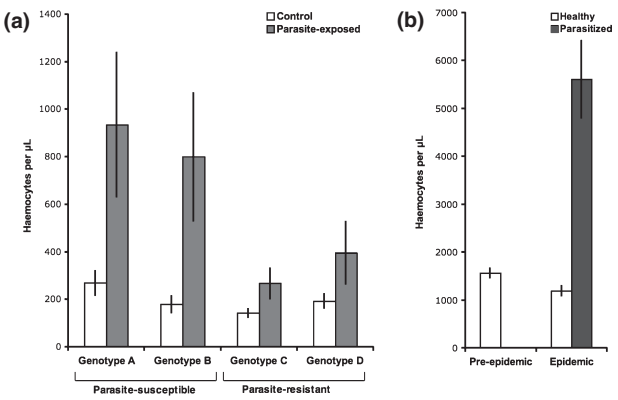


Adding power to these studies is the possibility to gain insight into genetic effects through controlled experimentation. Especially important for this experimentation is the fact that *Daphnia* are facultative parthenogens and can be cloned, which enables precise comparison of genetic backgrounds, or the study of different environments on replicates of the same genetic background. Experiments on susceptibility of *D. magna* to *P. ramosa* have revealed extensive genetic variation in both hosts and parasites (Ebert, Zschokke-Rohringer & Carius 1998; Little & Ebert 1999, 2000, 2001), including genetic specificity – that is, host genotype by parasite genotype interactions where the susceptibility of a host genotype is tightly dependent on the parasite strain to which it is exposed (Carius, Little & Ebert 2001). Similar 'context-dependence' has been revealed when hosts and parasites have been studied under different environmental conditions (genotype by environment interactions; Vale & Little 2009; Vale, Stjernman & Little 2008). Furthermore, the short generation time of *Daphnia* (~ 10 days) enables the study of real-time evolutionary responses to parasites (Little & Ebert 1999, Duncan & Little 2007, Zbinden, Haag & Ebert 2008). The *Daphnia* system is also unique for the accessibility of reconstruction of historical genetic changes via the resurrection of resting stages (Limburg & Weider 2002; Decaestecker *et al.* 2007).

Box 2. An immune measure for the dead

The study of a putative immune response in the crustacean *Daphnia* provides a simple yet striking example of the dangers of assuming that a stronger immune response represents greater host fitness. Immune responsiveness in *Daphnia* can be estimated by extracting a small amount of hemolymph and counting the abundant plasmatocytes (cells that appear to have phagocytic function). Different genotypes of *D. magna* show markedly different susceptibilities (Carius, Little & Ebert 2001) to the naturally coevolving bacterial pathogen *P. ramosa,* and recent work has revealed that immune responses are evident only in susceptible genotypes (see panel A below; Auld, Scholefield & Little 2010).

These data are from an experiment involving four long-term laboratory *Daphnia* lines for which resistance characteristics are well-established. Two lines are highly susceptible and two are highly resistant, and replicate hosts from each line were either exposed or not exposed (controls) to a spore suspension of *P. ramosa*. Compared to their controls, the susceptible genotypes showed a substantial increase in the number of circulating phagocytes in an 8-h period of exposure (data are a mean of six replicates studied from four time points: 2, 4, 6, and 8 h of exposure). An expanded data set on sixteen host genotypes largely confirmed this pattern (See Auld, Scholefield & Little 2010). Thus, *D. magna* may have a two-stage defence – a genetically determined barrier to parasite establishment, and a cellular response once establishment has begun. A strong immune response is a marker for susceptibility rather than resistance.



This result has since been borne out in field studies comparing hemocyte counts in naturally infected and uninfected hosts. Many *D. magna* populations experience summer epidemics of *P. ramosa,* and it can be shown that pre-epidemic hosts (which are of course not infected) have low hemocyte counts, comparable to healthy hosts during the epidemic period (panel B, above). The pre-epidemic samples represent a mean from three sampling dates in May, 2009, whilst the epidemic samples represent a mean of 13 sampling dates spread from June to October 2009 when *P. ramosa* was common in the population; S. K. J. R. Auld, A. L. Graham & T. J. Little, unpublished). *Pasteuria ramosa* sterilizes its host, and so hosts showing signs of infection (and thus high cell counts) will not directly contribute genes to the next generation. Thus, in the *D. magna–P. ramosa* interaction, a strong immune response is not associated with high fitness, but rather is tightly linked to being genetically dead.

methodologies borrowed from other branches of biology can disentangle relationships among the three traits. Our statistical advice is focused on rigorous exploration of relationships between host fitness and parasite density (i.e. evolutionary ecological tolerance).

WHEN MORE IS BLATANTLY NOT MORE: AN EXAMPLE

We begin by illustrating the benefits of three-trait data sets with an example, the crustacean *Daphnia magna* infected with the bacterium *Pasteuria ramosa* (Box 1). Several decades of both laboratory and field research have generated a deep understanding of the fitness consequences of parasitism in *D. magna* (Ebert 2005). Consequently, unlike ecoimmunological work in which hemocyte or white blood cell densities, for example, are quantified without knowledge of host fitness or relevant parasite biology, ecoimmunology of *D. magna* can be undertaken with extensive knowledge of potential evolutionary outcomes. Different host genotypes show markedly different susceptibilities to infection (Carius, Little & Ebert 2001), and yet after exposure, densities of responding hemocytes are highest in susceptible genotypes (Auld, Scholefield & Little 2010) (Box 2). Had hemocyte densities been measured in *D. magna* hosts without either prior knowledge of the system or knowledge of the infection status of individuals – that is, without the understanding that cellular responses are a marker for both genetic susceptibility and infection – we might have naively concluded that hosts with highest hemocyte densities would have the highest fitness. However, hosts with the most hemocytes actually tend to have the lowest fitness because they're infected with a sterilizing parasite! This example strikingly demonstrates that more is not necessarily more

Table 1. An array of ecoimmunological study designs which may be experimental or observational, performed in the field, the laboratory, or both

Design	Description	Possible measurements	Examples
1	Experimental: induce immune response to non-infectious agents in the field or lab	Host fitness *Immune response* *Density of natural parasites*	a, b, c
2	Experimental: infect with different doses of parasites, primarily in the lab	Host fitness *Immune response* *Parasite density*	d, e, f, g, h, i
3	Experimental: infect with different parasite genotypes, primarily in the lab	Host fitness *Immune response* *Parasite density*	j, k, l
4	Experimental: remove parasites in the field or lab	Host fitness *Immune response* Parasite density	m, n
5	Observational studies in the field	Host fitness Immune response Density of natural parasites	o, p

We argue that nearly any design would benefit from inclusion of immune response and parasite density measurements, to accompany measurement of host fitness. Possible measurements in plain text are not optional; items in *italics* are optional but recommended (see text). [a]Bonneaud *et al.* (2003), [b]Moret & Schmid-Hempel (2000), [c]Raberg & Stjernman (2003), [d]Ben-Ami, Ebert & Regoes (2010), [e]Bleay *et al.* (2007), [f]Lundgren & Thorpe (1966a), [g]Lundgren, Thorpe & Haskell (1966b), [h]Nol, Olsen & Rhyan (2009), [i]Xiao *et al.* (2005), [j]Carius, Little & Ebert (2001), [k]Grech, Watt & Read (2006), [l]Raberg, Sim & Read (2007), [m]Hudson, Dobson & Newborn (1998), [n]Pedersen & Greives (2008), [o]Norris, Anwar & Read (1994), [p]Stjernman, Raberg & Nilsson (2008).

in immunology, that well-studied host-parasite systems may be poised to make major contributions to ecoimmunology, and that host fitness and parasite densities [or other readouts of the efficacy of defense (Adamo 2004; Viney, Riley & Buchanan 2005)] must be measured alongside immune responses.

Three key traits in the context of ecoimmunological study designs

Various study designs enable ecoimmunologists to quantify the fitness consequences of immune responses (Table 1). Here, we highlight the role that the three focal measurements (host fitness, immune response magnitude, and parasite density) can play in each, to emphasize that more measurements per study rather than radically new study designs will go a long way to improving empirical ecoimmunology. We illustrate with examples, but have not attempted to be exhaustive.

We make several qualifications from the outset. First, fitness in terms of lifetime reproductive success is not easy to measure, but it remains an aspiration. Proxies such as annual survival, annual fecundity, or health must have demonstrated relevance to true fitness for the system under study. Secondly, the appropriate immunological and parasitological measurement(s) will vary greatly from system to system. We discuss how to promote selection of relevant parameters below. Thirdly, when quantification of parasite density is impossible but longitudinal studies are feasible (for instance, studies undertaken on free-ranging animal populations in the wild), duration of infection (e.g. days parasite positive) might in principle serve as the parasitological readout, though we know of no such studies to date. Fourthly, we caution that multiple independently-derived stocks of the parasite or immunostimulant may be needed, depending on the level of generalization desired. For instance, if only one strain of *Plasmodium* was used in an experiment [or indeed in years of experiments, as frequently observed in laboratory infection models (Viney 2006)], it is difficult to generalize to the fitness consequences of malaria as these may differ dramatically across strains/species. Finally, field and laboratory research have different weaknesses. In particular, field studies may be confounded by unknown exposure histories of hosts, whereas lab studies often use both host and parasite strains of restricted genetic diversity (Viney 2006). We believe that the most powerful ecoimmunological studies will combine such data (e.g. Box 2) and would encourage development of more systems that span the field-lab divide while quantifying host fitness, parasite density, and immune response magnitude.

DESIGN 1: EXPERIMENTS IN THE ABSENCE OF INFECTION

A common ecoimmunological study design involves non-infectious experimental manipulations such as injection with agents that spark immune responses [e.g. lipopolysaccharide (LPS) or vaccines; Design 1 in Table 1]. For example, injection of LPS into house sparrows followed by fitness measurements demonstrated that reproductive costs of immune responses may be compensated for by greater investment in the next clutch, among other mechanisms (Bonneaud *et al.* 2003). Injection of LPS into bumblebees demonstrated that survival costs of immune responses might only be expressed when resources are limited (Moret & Schmid-Hempel 2000). A key advantage of using parasite mimics rather than true infections is avoidance of the confounding influence of the mechanisms the parasite uses to circumvent immune responses (Huxham, Lackie & Mccorkindale 1989; Barnes & Siva-Jothy 2000).

Studies of Design 1 can be enriched by measurement of cellular or molecular immune responses. A particularly good example is the study of blue tits injected with tetanus-diphtheria vaccine, in which survival was monitored and vaccine-specific antibodies measured; a major finding was stabilizing selection on primary antibody responses to diphtheria (Raberg & Stjernman 2003). In other words, birds with either very weak or very strong responses to that antigen were unlikely to survive the winter. The birds probably do not experience diphtheria. Instead, the titre of vaccine-induced antibodies might be considered an index of overall immune responsiveness: weak responders are presumably prone to infectious diseases in general, hence their high mortality rate, while the high mortality rate of strong responders might arise from general or vaccine-induced costs of immunity (Raberg & Stjernman 2003).

For any study of Design 1, a difficulty is that the relevance of the induced response to an animal's ability to fight a real infection is rarely known (Adamo 2004; Staszewski & Boulinier 2004; Viney, Riley & Buchanan 2005; Martin, Weil & Nelson 2006). For example, does the magnitude of response to LPS predict responsiveness to live bacteria? Similar questions arise for the assumed relationship between diphtheria-specific antibodies and resistance to real infections of the blue tits described above. In principle, studies of Design 1 can be broadened to include measurement of the within-host densities of relevant parasites. This enables researchers to address whether strong responses to immunostimulants are correlated with lower prevalence or intensity of real infections (e.g. Lee *et al*. 2006). Indeed, we support calls for studies of Design 1 to provide 'functional readouts' (Viney, Riley & Buchanan 2005) or 'host resistance tests' (Adamo 2004) that lend insight into the ability of hosts to fight real infections.

DESIGNS 2–4: EXPERIMENTS IN WHICH INFECTIONS ARE ADDED OR REMOVED

The fitness consequences of strong immune responses probably depend upon the number and genotype of parasites with which a host is infected. Ecoimmunological experiments in which infections are added to or removed from hosts (Designs 2–4) aim to test that hypothesis. Just as data on immune response magnitude and/or parasite density make Design 1 studies more informative, the same applies to these designs.

Design 2, in which hosts are challenged with varying doses of live parasites, is commonplace in biomedical research, with the dose at which 50% of hosts can no longer prevent infection (infectious dose, ID_{50}) or survive infection (lethal dose, LD_{50}) serving as indices of host susceptibility. Indeed, dose-response experiments can reveal whether completely resistant host genotypes exist and, more generally, quantify the distribution of host susceptibility in a population (Ben-Ami, Ebert & Regoes 2010). When accompanied by immunological measurements, such experiments can also demonstrate whether there is a threshold number of parasites above which immune elements are induced, qualitatively altered, or else overcome (Bleay *et al*. 2007). If hosts die above a particular inoculating dose despite controlling parasite numbers, then disease may be due to a cytokine storm (uncontrolled production of signalling molecules, particularly by the innate immune system) or other immunopathology (Graham, Allen & Read 2005). A virulence factor of methicillin-resistant *Staphylococcus aureus* (MRSA) exhibits such dose-dependence: at low doses it induces protective innate immune responses, while at high doses it induces septic shock (Yoong & Pier 2010). The severity of other infections may entail similar dose-dependent shifts to immunopathology (e.g. among microparasites of vertebrate hosts (Schmid-Hempel & Frank 2007)). Such patterns have even been observed in invertebrates. In *D. magna*, for example, very high spore doses of *P. ramosa* may lead to drastic reductions in host fitness, even though parasite density often decreases with increasing dose (Ebert, Zschokke-Rohringer & Carius 2000). The benefits and costs of strong immune responses can therefore be obscured in studies of Design 2 unless parasite density and/or immune response magnitude are also measured as experimental outcomes.

Design 3, in which the experimenter varies the parasite genotype or species to which hosts are exposed, is indispensable for identification of genetic specificity of attack and defense that underpins so much of co-evolutionary theory (e.g. Carius, Little & Ebert 2001; Grech, Watt & Read 2006). Again, parasite density and immunological measurements aid interpretation by providing some mechanistic detail of within-host events. For example, whether the sickest hosts bear high parasite densities, cytokine storms, or both, can be shaped by parasite genotype (Long *et al*. 2008) and lead to different evolutionary trajectories (Day, Graham & Read 2007).

Design 3, accompanied by parasite density measurements, was used in the first declared test for tolerance in animals (Raberg, Sim & Read 2007). The study demonstrated that host genetic background conditioned how fitness (i.e. health of laboratory mice, in this case anaemia and cachexia) changed with increasing malaria parasite density. Mouse strains that experienced the shallowest declines in fitness with increasing parasite density were considered the most tolerant (Raberg, Sim & Read 2007). However, interpretational problems arise when parasite diversity and density are confounded – more generally, when density is not experimentally controlled – or when tolerance mechanisms are unknown, as discussed in detail below.

For a variety of ethical and logistical reasons, both Designs 2 and 3 may be difficult to apply outside the laboratory. For example, one may (rightly) be forbidden to infect wild animals experimentally. A possible exception would be to add ecoimmunological analysis onto epidemiological susceptibility studies such as those used to assess the potential for wild hosts to sustain transmission of zoonotic infections such as rickettsia, brucellosis, or monkeypox (Lundgren & Thorpe 1966a; Lundgren, Thorpe & Haskell 1966b; Xiao *et al*. 2005; Nol, Olsen & Rhyan 2009).

Better yet, Design 4, in which parasites are experimentally removed from wild hosts, is likely to be informative and

applicable across a wide variety of systems. Such experiments have been used to quantify how parasites (particularly nematodes) regulate host population size (Hudson, Dobson & Newborn 1998; Pedersen & Greives 2008), but the experiments can also reveal costs of parasitism borne by individuals and, in principle, the costs and benefits of immune responses (Pedersen 2005, Pedersen & Greives 2008). For example, following clearance of nematodes, measurements of the density of other parasites and the magnitude of subsequent immune responses can disentangle mechanisms of within-host interaction, as has been advocated for observational studies (Bradley & Jackson 2008). Design 4 seems a rich vein for future experimentation in ecoimmunology.

DESIGN 5: ECOIMMUNOLOGICAL OBSERVATIONS

When fitness measurements are coupled with data on parasite densities and/or immune response magnitude, purely observational studies can also yield rich insights (Norris, Anwar & Read 1994; Stjernman, Raberg & Nilsson 2008). For example, blue tits with both very low and very high densities of Apicomplexan parasites exhibit reduced overwinter survival (Stjernman, Raberg & Nilsson 2008). The data suggest that strong immune responses themselves are associated with mortality risk, while weak immune responses increase risk of mortality due to infection. Such an inference would be supported by evidence that birds with the lowest parasite densities exhibit the strongest parasite-specific immune responses. To our knowledge, such a data set does not yet exist, though the data of Raberg & Stjernman (2003) on vaccine-specific antibody and survival of blue tits (discussed above) lend support. Another observational ecoimmunological study – of the Soay sheep of St. Kilda – gains tremendous power via longitudinal tracking of survival, fecundity, and lifelong parasite densities of individual sheep (Clutton-Brock & Pemberton 2004). Immunological measurements have now demonstrated an association between antibody titres and the ability of sheep to resist nematodes (Coltman *et al.* 2001) and to survive harsh winters (Graham *et al.* in press).

One problem with observational studies is that a wild host that bears few parasites might not necessarily be resistant to infection, but might instead have avoided exposure (Sheldon & Verhulst 1996). It is sometimes possible to pair observational data with experiments that distinguish these distinct causes of parasite density – for example, in the case of potential environmental influences on both exposure and susceptibility of amphibians to trematode infections (Rohr *et al.* 2008) or dose-response experiments on *D. magna* (Ben-Ami, Ebert & Regoes 2010). However, when controlled experiments are impossible, immune response measurements can also help to distinguish whether exposure or resistance best explains low parasite density (Bradley & Jackson 2008). For example, if helminth-free hosts bore high titres of IgE, then the inference of resistance to infection would be supported (Bradley & Jackson 2008).

WHICH PARASITES AND IMMUNE RESPONSES TO MEASURE?

The examples above highlight the value of measuring parasite density and/or immune response magnitude in the context of most ecoimmunology study designs, to 'open the black box' of mechanisms operating within hosts. For study systems that are not yet well characterized, exactly what to measure may not be obvious – for example, if the entire parasite fauna of the focal host species is unknown, or if the type of immune response required to kill a particular parasite is difficult to extract from the encyclopaedia of immunological possibilities. We suggest that opening the black box enough to permit evolutionary ecological inference does not require hugely specialized knowledge of parasitology and immunology. It does require dedication, however, and a willingness to think beyond LPS, phytohemagglutinin (PHA), sheep red blood cells (sRBC), and other tried and true but nonetheless limited workhorses of ecoimmunology (Adamo 2004; Viney, Riley & Buchanan 2005; Martin, Weil & Nelson 2006).

Of course, the final decision of what to measure hinges on both relevance and feasibility. Relevant parasites are likely to be the most prevalent/abundant in the environment or in hosts, though they might also be parasites that are rare but cause severe disease (Grenfell & Dobson 1995). The over 130 years of publications in parasitology and infection biology may provide excellent clues on what parasite(s) to measure, especially if related host species have received attention. Feasible parasites are those for whom samples can be obtained, ideally noninvasively, and for whom density (or at least prevalence) can be quantified. Blood and faeces are good places to begin looking for parasites of vertebrates (or invertebrates; e.g. Lazzaro, Sackton & Clark 2006), and for parasites such as helminths and protozoa, little more than vital stains and basic microscopes might be required. PCR-based techniques can make the detection of parasites feasible from almost any tissue.

The relevant immune response to measure often follows on from the relevant parasites, because the immune system to a large extent must tailor parasite killing mechanisms to the size, location (intracellular vs. extracellular, as well as gut vs. blood vs. other anatomical location), and route of entry of parasites (Schmid-Hempel 2005; Weaver & Murphy 2007). Thus, for example, if nematodes are prevalent and deadly, as among the Soay sheep, then it makes sense to target nematode-specific IgA for measurement (Clutton-Brock & Pemberton 2004). If instead blood-borne Apicomplexans are prevalent and deadly, as among Hawaiian birds, then it would be better to measure malaria-specific cytophilic IgY (Lee *et al.* 2006). Targeted measurement of cellular responses in *D. magna* (Box 2) and other invertebrates makes sense because many innate immune responses are based primarily on phagocytic cells (e.g. Elrod-Erickson, Mishra & Schneider (2000). These cells also generate non-specific reactive oxygen and nitrogen species or phenoloxidase that destroy pathogens and can

also be measured (Rolff & Siva-Jothy 2003; Rivero 2006). In vertebrates, it can be also be informative to measure non-specific molecules such as complement or natural antibody (Adamo 2004). Feasibility for immunological measurements is determined by the availability or development of appropriate tools for each host species (Bradley & Jackson 2008). We do not underestimate the difficulty of this enterprise (Matson *et al*. 2006), but we also feel that the benefits of working with real parasites and real immune responses (see also Martin, Weil & Nelson 2006) cannot be overstated.

Relationships among traits

Of course, choosing the right parasites and immune responses to measure is just one step. Next, the causal relationships among traits must be considered. This issue was highlighted at the beginning of this article with the *Daphnia* example, where a large immune response indicates susceptibility. The general point is that an immune response of a particular magnitude can either be a cause OR a consequence of a particular parasite density. For example, a high antigen-specific antibody titre can be indicative of resistance to infection by parasites bearing that antigen, but it can also indicate persistence of that antigen in the host.

Measuring both parasites and relevant immune responses is key to resolving directionality, because a negative correlation between them is predicted if immune responses cause resistance, whereas a positive correlation is predicted if immune responses merely reflect antigen load or present parasite density (see also Sheldon & Verhulst 1996; Lee *et al*. 2006, Whiteman *et al*. 2006; Bradley & Jackson 2008). If the magnitude of an appropriate effector immune response is uncorrelated with parasite density, then tolerance may be at work. That said, the magnitude and even the sign of these relationships can change over the course of infection. For example, early in infection, as immune responses ramp up, there may be a positive association between parasite densities and concentrations of immunological molecules. Later in infection, once most parasites have been cleared, the correlation may become negative. Controlled laboratory experiments will be critical to clarify these dynamics. Manipulative experiments in which immunological tools like monoclonal antibodies are used to alter levels of effector activity (e.g. Long *et al*. 2008) can reveal the extent to which particular immunological cells or molecules control parasite density in some systems. Longitudinal field studies – for example, of the dynamics of *Borrelia* exposure and *Borrelia*-specific antibodies in seabirds (Staszewski *et al*. 2007) – may also be informative. Indeed, theoretical groundwork for exploring relationships between parasite density and immune response magnitude has been laid, but data are lacking (Fenton & Perkins 2010).

Another key relationship in our triad of recommended traits is that between parasite density and host fitness. In the rest of this section, we outline analytical problems inherent in the study of this relationship and propose statistical solutions that should apply equally to relationships among all traits in the triad.

DEFINING TOLERANCE

Evolutionary ecologists have come to call the relationship between host fitness and parasite density tolerance (Raberg, Sim & Read 2007; Ayres & Schneider 2008, Ayres & Schneider 2009, Pagan, Alonso-Blanco & Garcia-Arenal 2009; Raberg, Graham & Read 2009). We note that this differs from the definition of tolerance in vertebrate immunology – that is, a lack of responsiveness to antigen that is actively maintained by cells of the immune system and essential to avoiding autoimmunity, for example (Abbas *et al*. 2004). However, we also note that cellular tolerance of parasite antigens can lead to organismal tolerance of parasites (Mills 2004), so the verbal definitions are not entirely at odds. The quantitative definition of tolerance poses greater challenges.

Tolerance according to the evolutionary ecological definition is the ability of hosts to limit the fitness costs of a given parasite density, but the quantitative definition has varied. In some theoretical (e.g. Roy & Kirchner (2000) and empirical (e.g. Ayres & Schneider 2008) studies, tolerance has been considered at a single parasite density, where two host genotypes bear the same number of parasites, but one genotype achieves higher fitness and is thus more tolerant of a given parasite density ['point tolerance' (Little *et al*. 2010)]. In other studies, tolerance has been considered a slope, quantifying how host fitness declines with increasing parasite density; more tolerant genotypes lose fitness less quickly as densities increase ['range tolerance' (Little *et al*. 2010)]. Genetic variation for range tolerance of rodent malaria was studied by Raberg, Sim & Read (2007), using an approach in line with studies of tolerance to herbivory (Tiffin & Rausher 1999; Simms 2000), though in plant studies the focus has been fitness (e.g. seed set) per unit of direct and measurable *damage* (e.g. leaf area lost due to herbivore chewing), while animal studies have thus far focused on fitness per parasite (see Baucom & de Roode in this issue). What is worrying is that alternative quantitative definitions – that is, point vs. range tolerance – can generate contradictory conclusions. For example, for two host genotypes that differ in range tolerance, their reaction norms will cross at some point in the range of parasite densities. If tolerance is estimated from relative fitness at a single parasite density, then the conclusion of which genotype is most tolerant depends upon where in the density range the underlying reaction norms cross, and the density at which point tolerance measurements are made (discussed in detail in Little *et al*. 2010).

Whenever possible (e.g. via dose-response experiments) range tolerance seems preferable to point tolerance to provide more comprehensive information about the fitness consequences of different defense strategies. However, range tolerance also raises complex analytical issues familiar to evolutionary biologists who study traits shaped by phenotypic plasticity or co-evolution.

HOW FITNESS DEPENDS ON PARASITE DENSITY: TOLERANCE AS PLASTICITY

If fitness is measured across a range of parasite densities, then range tolerance is directly analogous to the concept of plasticity under a linear reaction norm model (Scheiner 1993). It therefore seems likely that recent methodological advances in modelling phenotypic plasticity might usefully be applied to studies of tolerance. For example, fitness (W) of host genotype i at parasite density D might be modelled as:

$$W_{iD} = \mu + d.D + g_i + e \quad \text{Model 1}$$

where μ is the overall mean fitness, d is the average regression of fitness on parasite density (i.e. the mean range tolerance), g_i is the effect (relative to the overall mean) of having genotype i, and e is a residual error. In practical terms, this model could be parameterized as a linear mixed effect model with g_i fitted as a random effect. This would allow estimation of the variance in g_i, which is properly interpreted as an estimate of the genetic variance for host fitness (under a parasite challenge) in the population from which tested host genotypes were drawn. However, Model 1 is only appropriate if the host genotypes differ in their average fitness (i.e. there is among-genotype variance in g_i) and not in the slopes of their regressions on parasite density. When this holds, estimates of point tolerance will yield the same fitness ranking of host genotypes regardless of the value of D at which they are tested [i.e. the reaction norms do not cross (Little *et al.* 2010)].

Alternatively, g_i may itself depend on D if range tolerances differ between genotypes. Assuming that a linear model of this dependence of g_i on D is appropriate we should then expand our model such that:

$$W_{iD} = \mu + d.D + g_{\text{int}.i} + g_{\text{slope}.i}.\ D + e \quad \text{Model 2}$$

where $g_{\text{int}.i}$ is a genotype-specific effect on mean host fitness (relative to μ) while $g_{\text{slope}.i}$ is a genotype-specific effect on the regression of host fitness on parasite density. This model could be parameterized by adding a genotype by parasite density term to the random effect structure of the mixed model in a random regression (so-called because the regression is contained with the random effect structure of the model). This approach is increasingly being used to model reaction norms across environmental gradients (Nussey, Wilson & Brommer 2007). On a practical note, it is often useful to zero-centre the D axis such that the estimate of variance in g_{int} can be interpreted as the genetic variance for fitness under an average parasite density (i.e. when $D = 0$).

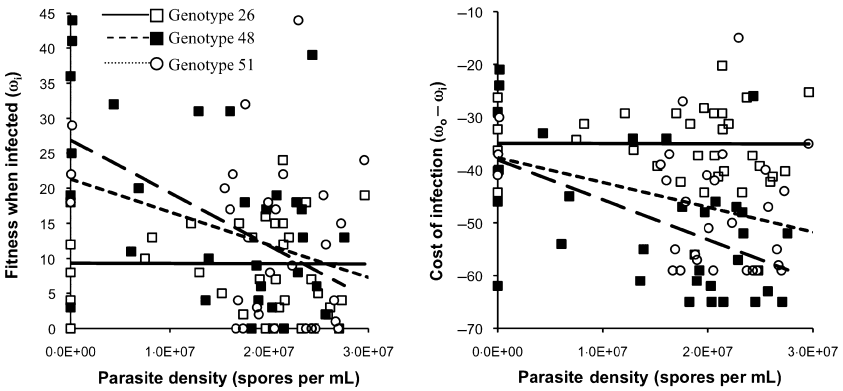
However, the key point to take from Model 2 is that, as outlined verbally by Little *et al.* (2010), if genotypes differ in their reaction norm slopes (i.e. there is variance in g_{slope}) then we expect the relative fitness ranking of different genotypes to change with D (though not necessarily within the range of parasite densities tested, nor within a biologically relevant range). A second point to note is that by formulating Model 2 as a mixed effect model a researcher can – and should – explicitly account for the covariance between reaction norm slopes and intercepts. Failure to account for this covariance can generate biologically misleading results because the information needed for evolutionary inference will often be influenced by the way in which tolerance relates to fitness in the absence of infection (the intercept). Host genotypes will almost certainly show fitness differences in the absence of infection – that is, genetically determined life-history variation is common (Stearns 1992). These differences may be linked to variation in the traits that contribute to defense via pleiotropy, as follows. One scenario is where defense against parasites is traded-off against vigor – that is, where a host possessing an allele that confers more potent defense is less fit than other genotypes when parasites are not around. But even in the absence of trade-offs, measurement of fitness of both infected and uninfected hosts is key, and *a priori* omission of intercepts from analyses of range tolerance (e.g. Raberg, Sim & Read 2007) may greatly limit inference about evolutionary outcomes. In Box 3, we illustrate this using data from the *D. magna–P. ramosa* system. Measuring the intercept of the reaction norm should be routine in laboratory studies of tolerance in which it is feasible to include control animals that are unexposed to the focal infection.

HOW FITNESS DEPENDS ON PARASITE DENSITY: CAUSATION AND CO-EVOLUTION

Another concern about the study of range tolerance in animals centres on the issue of causation. This is because parasite density, host fitness, and even immune responses are likely to be under the joint control of the host and the parasite. For example, leaving aside environmental effects on exposure, parasite density within a host is the result of the parasite's intrinsic replication rate and the host's ability to kill parasites. Immune response magnitude is the result of the host's intrinsic responsiveness and the immunogenicity of, or immunosuppression by, the parasite. Finally, host fitness when infected depends on all of the above, plus parasite virulence, plus host tolerance (Little *et al.* 2010)! Parasite growth within hosts is therefore difficult to experimentally control [even when controlling for genotype-by-genotype or genotype-by-environment interactions (e.g. Box 1)]. This problem may not apply to macroparasites such as helminths that do not replicate within the host (Bleay *et al.* 2007) or that have resting stages (Stopper *et al.* 2002), and thus their densities can be largely controlled via inoculating dose, but the problem certainly pervades the study of microparasites. Consequently, microparasite density at time t can be considered an uncontrolled outcome of the experiment, as opposed to an explanatory variable in the sense of regression or analysis of covariance (Sokal & Rohlf 1995). Here, it is not possible to disentangle whether parasite density determines host health (and by extension, host fitness), or if host health determines parasite density: they fundamentally confound each other.

Box 3. Inferring evolution from linear relationships between parasite density and host fitness

Many empirical studies have considered the linear relationship between parasite density (within hosts) and a measure of host fitness. Although a linear relationship may not always be representative, it can be adequate over some ranges of parasite density. But even in these cases, there are nuances to consider, in particular regarding the role played by host fitness in the absence of infection, that is, the *y*-intercept. Perhaps the majority of studies on the relationship between parasite density and host fitness have sought to gain insight into parasite evolution (evolution of virulence studies; e.g. De Roode *et al*. 2005), and thus the measurement of host traits in the absence of infection has been understandably ignored. Similarly, tolerance studies might not consider fitness in the absence of infection (which we call ω_0) because tolerance, by definition, does not include ω_0. And yet, it is difficult to make inference about selection on tolerance when ω_0 is not measured. First, the fitness of a particular genotype will be determined by both ω_0 and its fitness across parasite densities. These two components of fitness may not be independent due to pleiotropic effects, but even when they are, jointly considering how they covary sheds light on what the rank fitnesses of different genotypes might be. Secondly, it may not be realistic to estimate ω_0 from a *y*-intercept of a parasite density-host fitness relationship in a study that has not directly measured ω_0.



To highlight these points, we present the results of an experiment that exposed the crustacean *D. magna* to the bacteria *P. ramosa* (see Box 1 and Appendix S1, Supporting information). Fifteen replicates of each of twelve host genotypes were exposed to the parasite, and the number of offspring produced by infected hosts was counted. Later, infected hosts were killed and the density of parasite transmission spores (per mL of host tissue) was estimated. Thus, we gained the data necessary to plot parasite density (within-hosts) against host fitness (in this case measures of fecundity). For convenience, we use ω_i to represent the 'fitness of infected hosts'. We also measured the reproductive output of control hosts, that is, the fitness of those hosts not exposed to the parasite, ω_0. Full experimental details are presented in Supporting information.

We studied the relationship between parasite density and host fitness in two ways. First, we studied only 'fitness of infected hosts', ω_i. Secondly, we incorporated host fitness in the absence of infection (ω_0), by studying simply $\omega_0 - \omega_i$. As ω_0 represents what hosts can achieve in the absence of infection, $\omega_0 - \omega_i$. is the cost of infection. The two graphs above compare fitness when infected (ω_i) and the cost of infection ($\omega_0 - \omega_i$) across parasite densities.

For clarity, results for only three of the 12 genotypes are depicted, and we multiplied the cost of infection by (−)1 so that higher values represent greater fitness, making the two graphs visually comparable. Of particular note here is how inference regarding which is the most fit genotype changes depending on the fitness measure used. When examining only ω_i (fitness when infected), left graph, the genotype (26) that is the most tolerant in terms of range tolerance (i.e. shows the flattest slope) is less fit than the less range tolerant genotypes, except at the very highest parasite densities. However, in looking at the cost of infection, that is, once the response variable incorporates information about fitness in the absence of infection ($\omega_0 - \omega_i$, right graph), the most tolerant genotype is also potentially the most fit. The other two genotypes also switch their rank order of fitness over most, but not all parasite densities. The reason for these differences is that ω_0 is not accurately estimated by the relationship between parasite density and ω_i. Indeed, including all 12 host genotypes, a correlation between the *y*-axis intercept, as estimated from linear functions such as those in the left graph, shows no relationship with the actually measured fitness in the absence of infection ω_0 (spearman $\rho = -0{\cdot}2028$, $P = 0{\cdot}51$). How the cost of infection will ultimately determine the winner of a competition between genotypes will be determined by the local frequency of epidemics.

Thus, host genetic variation for range tolerance represents how genotypes differ in the strength of a relationship (typically studied as a regression) between parasite density and health/fitness, but it is difficult to say why. This becomes pertinent when considering the process of natural selection: without understanding the cause of differences in the

strength of relationships, it is not clear what trait is being selected upon and what evolutionary response to selection we should expect to see. For instance, it is possible that molecular mechanisms of tolerance control the relationship. If, for example, an immunological mechanism [e.g. anti-toxin or anti-inflammatory molecules (Raberg, Graham & Read 2009)] can be shown to alleviate disease severity as parasite numbers increase, it becomes more straightforward to interpret how natural selection will act on variation in range tolerance. This is because the immunological mechanism might then be understood to be the trait subject to natural selection. In the absence of such a mechanism, however, it is equally possible that different genotypes are just more or less sensitive to the laboratory environment, leading to differences in health and then parasite load. Here, we run the risk of confounding tolerance of the environment with tolerance of the infection. Interpreting the relationship between parasite density and fitness requires considerable caution because it is explicitly the product of two interdependent measures.

Similar issues have been discussed in other fields, and seem dangerous to ignore. For example, Ridley (1988), in his treatment of the benefits of multiple mating in insects, contrasted 'experimental comparisons' (with controlled explanatory variables), with 'non-experimental comparisons' (the uncontrolled, descriptive approach). In the latter kind of study, the risk is that experimental individuals in a sense self-select which treatments groups (once mated, twice mated, etc.) they are in, perhaps due to their condition. This self-selection may seem justified if randomly allocating individuals to treatments beforehand (the correct approach) entails significant loss of experimental subjects if some proportion of replicates fail to complete the required number of matings. However, it has become clear that different conclusions have been drawn about insect mating behaviour depending on the method used (Ridley 1988); see also Torres-Vila, Rodriguez-Molina & Jennions (2004). The similarities to experimental infection studies are obvious, as hosts (and parasites) may 'self-select' how a given dose turns into a given parasite density. Although this imposes a constraint on experimental design and inference, it cannot be ignored.

With respect to the study of tolerance, we gain some traction on the problem by applying a range of parasite doses, although (as outlined above) dose will often show complex relationships with microparasite density – for instance it may be highly nonlinear [e.g. *Pasteuria* in *Daphnia* (Ebert, Zschokke-Rohringer & Carius 2000)], or dose may influence the timing but not the magnitude of peak parasite density [e.g. *Plasmodium* in *Mus* (Timms *et al*. 2001)]. Alternatively, it may be feasible to inoculate with a single parasite dose and then apply a range of subcurative doses of an anti-parasite drug, although we know of no examples of this approach in which tolerance was quantified and we would caution that various potential confounding effects, especially if the drug has a direct impact on host health or if initial dose is all that matters, require careful thought. Injection of LPS or heat-killed bacteria might be informative for quantifying tolerance of septic shock. Lastly, there is the potential to use a range of parasite genotypes that differ in the density they tend to reach (Raberg, Sim & Read 2007), although this tendency would have to be independent of host genotype – that is, host genotype by parasite genotype interactions (*sensu*; Carius, Little & Ebert 2001) would confound this approach. Overall, statistical approaches that can account for the interdependency of measures in ecoimmunological data sets seem warranted.

BEYOND REGRESSION-BASED APPROACHES

In our discussion of phenotypic plasticity, we highlighted ways in which statistical methods such as random regression might benefit ecoimmunology. However, our advocacy of such methods should not distract from the fact that important, but largely unrecognized, statistical issues arise when neither experimental control of parasite density nor investigation into mechanism are feasible. First, if parasite density is not experimentally controlled it will necessarily be measured with error that is typically unaccounted for in regression based analyses of tolerance. Under simple (type I) linear regression, measurement error in the explanatory variable will lead to underestimation of the magnitude of the slope (i.e. overestimate tolerance) (Sokal & Rohlf 1995). This problem could be avoided by use of type II or major axis regression. However, a second issue is that any regression model specifies and assumes a uni-directional cause-effect relationship between parasite density (the independent variable) and host fitness (the response). As outlined above, however, there are good biological reasons to expect that the relationship to be bi-directional. Statistical models must always make simplifying assumptions and we do not suggest that regression be abandoned, only that violated assumptions be more widely recognized and that alternative, complementary types of analyses warrant consideration. For instance, while correlation can never prove causation, path analysis and structural equation modelling might allow different models of causal relationships between the measured host and parasite processes to be considered (and in some cases statistically compared) (Mitchell 1992; Shipley 1997).

Alternatively, there is considerable logic in choosing to treat both parasite density and host fitness as response variables in a bivariate analysis. For instance, using a bivariate mixed model (Lynch & Walsh 1998), the observed covariance between parasite density (D) and host fitness (W) can be modelled and decomposed into components attributable to factors of biological interest (e.g. host genotype or source population) and experimental design (e.g. block). For example, by fitting host genotype as a random effect (and assuming that repeated observations on each genotype are available) the total variance (V) in a trait (x) can be decomposed into a portion attributable to host genotype and a residual component (attributable to unmodelled environmental effects and measurement error). In a bivariate model the total variance–covariance matrix for two traits can be similarly partitioned such that:

$$\mathbf{P} = \mathbf{G} + \mathbf{R}$$

where **P** is the phenotypic variance–covariance matrix between n (in this case 2) traits, **R** is the matrix of residuals (usually interpreted as environmental effects), and **G** is the genetic covariance matrix

$$\mathbf{G} = \begin{bmatrix} V_{G(W)} & \mathrm{COV}_{G(WD)} \\ \mathrm{COV}_{G(WD)} & V_{G(D)} \end{bmatrix}$$

where $V_{G(W)}$ and $V_{G(D)}$ are the among-host genotype (i.e. genetic) variances for fitness and parasite density, respectively, while $\mathrm{COV}_{G(WD)}$ is the genetic covariance term. If so desired these parameters could be rescaled to yield the heritabilities of W and D (seen as traits of the host) as well as the genetic correlation, although it should be noted that these will typically be broad-sense (as opposed to additive) genetic parameters if clonal replicates are used. Moreover, these models are not limited to the study of genetic correlations, and they are not limited to bivariate. Researchers could include all response variables in a single model, and can then extract almost any pairwise linear relationships, including regressions, that are of interest.

This approach also provides an unexploited link to quantitative genetic models of trait evolution, since the genetic covariance between a trait and (relative) fitness actually provides an unbiased prediction of the expected selection response (Robertson 1966; Morrissey, Kruuk and Wilson, in press). A simple corollary of this is that even if there is an association between host fitness and parasite density, evolution of the host mechanisms for controlling the parasite density is not expected if $\mathrm{COV}_{G(WB)} = 0$ and all covariance arises from environmental sources of covariance (portioned into **R**). Given suitable data, further partitioning of **P** is readily achieved by addition of further random effects. While additional random effects may certainly be used to test specific hypothesized sources of environmental covariance between D and W (e.g. maternal effects, host cage effects), a second genetic covariance structure may be estimated in the event that multiple parasite genotypes were used (with replicate observations for each). Thus, it is possible to model W and D as traits that vary, and covary, as a consequence of interacting host and parasite genotypes, and to estimate the relative contributions of each to observed (co)variance. In this way genetic control of W and D need not be assumed to lie with either the host or the parasite, but rather can be influenced by both. We encourage ecoimmunologists to explore these approaches in more detail across a range of organisms.

Optimal studies of optimal immunity

With this article, we suggest three primary improvements to the empirical framework for ecoimmunology. In brief, we urge researchers to make more measurements, to choose them wisely, and to analyse them using some of the statistical techniques that have permeated other fields and are recommended above. The additional measurements (immune response magnitude and parasite density, to complement host fitness in the context of various study designs; Table 1) help to dissect important details of within-host dynamics – for example, are hosts more likely to die of high parasite densities or of immunopathology (Graham, Allen & Read 2005)? Wise choice of which immune elements and parasites to measure ensures relevance to fitness but requires basic knowledge of the infection biology of the target hosts or of related, well-investigated model systems (Bradley & Jackson 2008). Finally, statistical methods used in other branches of evolutionary biology appear more appropriate than current methods for dealing with inherent issues in ecoimmunological data sets (e.g. bi-directional causal relationships). We provide preliminary statistical advice for studying tolerance, but the suggested methods should apply to any data on host fitness, parasite density and/or immune responses. Together, our suggestions promote robust quantification and interpretation of fitness consequences of immune responses. We hope to prompt researchers to tailor suggestions according to what is most reasonable and appropriate for their systems and research goals. Most studies are imperfect (including those of the authors), but with steps such as those explored here, studies of ecoimmunology and optimal immunity (Viney, Riley & Buchanan 2005) can better approximate perfection.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Appendix S1. Details of methods and statistical analyses for data in Box 3.

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